Immunomodulation of the IgE dependent immune response by docosahexaenoic acid

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Zusammenfassung

Weltweit wird ein Prävalenzanstieg Typ I allergischer Erkrankungen beobachtet. Vor dem Hintergrund einer genetischen Prädisposition tragen verschiedene endogene und exogene Einflüsse zur Pathogenese dieser Immunglobulin E (IgE)-vermittelten Krankheiten bei. Westliche Lebensstilelemente, insbesondere die Ernährungsgewohnheiten, gelten als Risikofaktoren für die Ausprägung allergischer Erkrankungen. Die westliche Ernährung ist durch eine Zunahme von omega-6 (n-6) mehrfach ungesättigter Fettsäuren (polyunsaturated fatty acids, PUFA) sowie eine Abnahme von n-3 PUFA gekennzeichnet. Fischöl, welches reich an n-3 PUFA ist, ist für seine antiinflammatorischen Effekte bekannt. Ebenso wurde die n-3 PUFA Docosahexaensäure (DHA) bei verschiedenen chronisch-entzündlichen Erkrankungen erfolgreich therapeutisch eingesetzt. Allerdings sind die zugrunde liegenden Wirkungsmechanismen nicht vollständig aufgeklärt.

Im ersten Teil der Arbeit wurde der molekulare Mechanismus der DHA-vermittelten Hemmung der IgE-Produktion in humanen B-Zellen untersucht. Neben einer dosisabhängigen Reduktion der anti-CD40/ Interleukin (IL)-4-induzierten IgE-Produktion führte die Behandlung von B-Zellen mit DHA zu einer verminderten Differenzierung IgE-produzierender Plasmazellen. Dieser DHA-vermittelte Effekt steht in kausalem Zusammenhang zu einer direkten Hemmung des Klassenwechselprozesses zu IgE auf dem Niveau des epsilon-Keimbahntranskriptes (εGLT) sowie der Aktivierungsinduzierten (Cytidin)-Desaminase (AID). Weitere Analysen der beteiligten Signaltransduktionswege ergaben, dass DHA sowohl die IL-4-abhängige Phosphorylierung des Signaltransduktors und -aktivators der Transkription 6 (STAT6) als auch die CD40-vermittelte Translokation des nukleären Transkriptionsfaktors kappa B (NFκB) p50 in den Zellkern hemmt.

Um zu überprüfen, ob die ex vivo gewonnenen Erkenntnisse auch in vivo relevant sind, wurde in einer randomisierten, kontrollierten Doppelblindstudie die Wirksamkeit einer Supplementierung mit hochdosierter DHA bei Patienten mit atopischem Ekzem hinsichtlich klinischer und auch immunologischer Parameter untersucht. In dieser Untersuchung führte DHA, aber nicht das Kontrollsupplement zu einer signifikanten Reduktion des Schweregrades der Erkrankung. Ferner wurde eine signifikant verminderte IgE-Produktion anti-CD40/IL-4-stimulierter Blutzellen von DHA supplementierten Patienten festgestellt. Wohingegen die Serum-IgE-Spiegel nicht beeinflusst waren. Parallel wiesen DHA-supplementierte Patienten eine erhöhte Konzentration an n-3 PUFA sowie ein reduziertes n-6/n-3-PUFA-Verhältnis im Plasma auf. Diese Beobachtungen unterstützen die Resultate des oben genannten zellbiologischen Teils und verdeutlichen die klinische Relevanz dieser Daten.

Im dritten Teil der Arbeit wurden die lokalen Prozesse in der Haut nach DHA-Supplementierung untersucht. Hierzu wurde ein Mausmodell eingesetzt, da es aus ethischen Gründen nur begrenzt möglich ist, Hautproben von Patienten zu analysieren. In Übereinstimmung mit den Ergebnissen der klinischen Untersuchung führte die orale DHA-Supplementierung auch zu einem verbesserten klinischen Bild der proteininduzierten Dermatitis. Die Reduktion der Symptomstärke und der damit einhergehende verminderte klinische Schweregrad des Ekzems waren mit der verringerten Zahl dermaler $CD8^+$ T-Zellen verbunden, indessen waren andere Parameter wie Mastzell- und $CD4^+$ T-Zellzahlen sowie Epidermisdicke nicht beeinflusst. Da auch im Mausmodell die Serum-IgE-Konzentration nicht durch die orale DHA-Verabreichung beeinflusst wurde, wirkt DHA hier vermutlich primär lokal als systemisch immunmodulierend.

Durch die Fähigkeit von DHA in den IgE-Klassenwechsel in B-Zellen einzugreifen, stellt die orale Supplementierung mit DHA eine mögliche präventive Maßnahme gegenüber Typ I allergischen Erkrankungen dar. Weiterhin verdeutlichen diese Daten, dass DHA den Schweregrad des atopischen Ekzems durch eine positive Beeinflussung lokaler inflammatorischer Prozesse signifikant verbessern kann. Um diätetische DHA bestmöglich als therapeutisches Instrument im Zusammenhang mit allergischen Erkrankungen einsetzen zu können, muss allerdings die Supplementierung hinsichtlich des Zeitpunktes sowie der Dosierung optimiert werden.

Abstract

The prevalence of type I allergic diseases have increased worldwide. Based on genetic susceptibility diverse endogenous and exogenous factors contribute to the pathogenesis of these immunoglobulin (Ig) E mediated disorders. The modern life style is hypothesised to be one risk factor for allergic diseases. Thereby the Westernised diet is characterised by an increasing consumption of omega-6 (n-6) polyunsaturated fatty acids (PUFA) and a decreasing intake of n-3 PUFA. Fish oil, rich in n-3 PUFA, exerts a wide range of antiinflammatory effects. Docosahexaenoic acid (DHA) is one major n-3 PUFA of fish oil and has been reported to be beneficial in different chronic and inflammatory diseases as well. However, the underlying mechanisms of its action are not completely understood.

The molecular mechanisms of DHA on IgE production in human B cells were examined in the first part of this thesis. DHA inhibited IgE production and the differentiation of IgE secreting cells in a dose-dependent manner. This inhibition was mediated through direct inhibition of the immunoglobulin isotype switching process, which was detected by decreased epsilon germline transcript (εGLT) and activation induced (cytidin) desaminase (AID) transcription. Analysis of involved signalling pathways revealed that DHA caused both an inhibition of interleukin (IL)-4 driven signal transducer and activator of transcription 6 (STAT6) phosphorylation but also a reduced nuclear factor kappa B (NFκB) p50 translocation into the nucleus upon anti-CD40 stimulation.

Next it was verified whether the ex vivo findings are relevant in vivo as well. In a randomised, double bind, controlled clinical study the efficacy of high-dose DHA supplementation in atopic eczema was determined and thereby the impact on clinical as well as immunological parameters investigated. In the DHA treated, but not in control group a significant clinical improvement of atopic eczema in terms of severity was observed. Additionally, in DHA group a significant reduction of anti-CD40/IL-4 mediated IgE synthesis of peripheral blood cells was detected whereas serum IgE concentrations remained unchanged. Furthermore, the DHA group showed an increase of plasma n-3 PUFA and a decrease of n-6/n-3 PUFA ratio. These observations support the findings from the first part of this work and point to their potential biological relevance.

The third part of this work deals with the clinical impact of oral DHA administration on allergen induced dermatitis. Due to ethical considerations analysis of skin biopsies from patients is limited. Therefore a mouse model was used. The obtained data correspond to the other results. Analysis of the local mechanisms of clinical improvement implicated a favourable

modulation of the cellular immune response by DHA. The reduced clinical skin score was associated with a decreased number of dermal $CD8^+$ T cells, whereas other parameters like epidermis thickening, $CD4^+$ and mast cell numbers were not affected. However, the serum IgE values remained unchanged indicating that DHA might rather act locally than systemically on an established allergic response.

Taken together the results of this thesis indicate that dietary DHA may be effective in prevention of type I allergic diseases by interference with the IgE switching process. Additionally, DHA has been shown to improve the clinical outcome of atopic eczema by having a positive impact on local inflammatory processes. Dietary DHA might be a potential therapeutical tool for dietary management of IgE mediated diseases, like atopic eczema. However, further research in order to reveal the best time-point and optimal dose of DHA application is still necessary.

Schlagwörter:

Docosahexaensäure, Mehrfach ungesättigte Fettsäuren, Allergie, IgE

Keywords:

Docosahexaenoic acid, Polyunsaturated fatty acids, Allergy, IgE

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1 Introduction

1.1 Type I allergic reactions

Whereas the Western world was experiencing a dramatic increase in the prevalence of allergic diseases during the last 30 to 40 years of the previous century, a similar tendency can be currently seen in fast developing countries such as India and China. Even in Arctic region the same trend is detected with a doubling of sensitisation rates in only 11 years [1].

Allergies are inappropriate or exaggerated reactions of the immune system to substances that are usually harmless. Allergy inducing substances are called allergens. Common indications of allergy may include sneezing, itching and skin rashes [2].

Immediate allergic reactions are manifested as anaphylaxis, rhinoconjunctivitis allergica and allergic asthma [3]. These immunoglobulin (Ig) E mediated processes are initiated by the first contact between immune system and allergen, the sensitisation phase. Thereby, the allergen is internalised and processed by antigen presenting cells (APC; like macrophages, dendritic cells [DC], Langerhans cells [LC], B cells). Peptides derived from allergens are presented via major histocompatibility complex II (MHC II) to naïve $CD4^+$ cells (T_H0) leading to T cell activation and differentiation into T_H2 cells. Signalling molecules of T_H2 cells, like CD40 ligand (CD40L, CD154) and interleukin (IL)-4, activate B cells to proliferate and differentiate to IgE secreting plasma cells [4,5]. The subsequently secreted allergen specific IgE binds to its high affinity receptor (FcεRI) on granule containing cells, like mast cells (MC) and basophils. Repeated exposure leads to cross linking of receptor bound IgE by polyvalent allergens, what triggers the degranulation of MC and basophils. The release of mediators, like histamine and prostaglandins (PG), elicits the symptoms of the immediate allergic reaction characterised by increased vascular permeability and smooth muscle contraction. The late phase is caused by leukotrienes (LT), chemokines and cytokines. The second phase of smooth muscle contraction and tissue remodelling takes place. Chemokines and local cytokine pattern recruit macrophages and neutrophils, but also eosinophils and T_H2 cells to sites of inflammation. Eosinophilic inflammation is supported by T_H2 cell derived IL-5. A sustained allergen exposure with a persisting immune reaction can lead to a chronic inflammation, hallmarked by hyperreactivity and damage of the affected tissue [6].

1.1.1 Mechanisms and molecular regulation of IgE production

IgE is the major player of type I hypersensitivities. The heavy chain constant (C_H) region domains of IgE are encoded by a single gene, the strictly regulated Cε gene [7]. In serum of

non-atopic individuals, IgE is found in very low concentrations ranging between 1 and 400 ng/mL [7]. This is mainly adjusted by a tight regulation of gene expression and protein stability [7]. The rapid turnover and the short half-life of 2.5 days contribute to the low serum IgE levels [1]. It has been considered that a high level of serum IgE comes along with high IgE production. However, increasing IgE levels lead to diminished IgE catabolism. Thus, half-life arises threefold when IgE levels proceed from 0.07 to 35 µg/mL [8]. Furthermore, when bound to its high affinity receptor on MC, half-life of IgE is increased up to 8 to 14 days [8]. Interestingly, in healthy individuals about 67 % of IgE is found extravascularly compared to 48 % in patients with severe atopic diseases [8].

According to their C_H regions immunoglobulins can be categorised into nine isotypes: IgA_1, IgA_2, IgD, IgE, IgG_1, IgG_2, IgG_3, IgG_4 and IgM. Except IgM and IgD, all other isotypes result from class switch recombination (CSR). Thereby, the specificity defining variable region is molecularly linked with the constant (C) regions of different isotypes and distinct effector functions [6].

Switching to all isotypes is commonly regulated at the germline transcription of C_H genes and the induction of activation induced (cytidine) desaminase (AID) expression. Switching from naïve B cell expressing IgM and IgD to IgE can occur directly or sequentially via IgG. This process is induced by two signals: antigen specific T cell interaction with B cell via CD40L/CD40 and IL-4 secreted by activated T cells [5].

IL-4 and IL-13 exert their biological effects by binding to the IL-4 receptor (IL-4R) complex, which consists of two subunits (Figure 1). Type I IL-4R complexes, composed of IL-4Rα chain and γc chain, bind IL-4 but not IL-13. IL-4 and IL-13 can bind to Type II IL-4R complexes, which are composed of IL-4Rα and IL-13Rα1. Both receptor complexes amplify their signals through IL-4Rα. A second IL-13 receptor, IL-13Rα2, binds to IL-13 exclusively and serves as a decoy receptor. IL-4 binding mediates receptor heterodimerisation and activation of cytoplasmic protein tyrosine kinases called Janus activated kinases (JAK) which are constitutively associated with IL-4Rα (JAK1) and γc chain (JAK3) [9,10].

Tyrosine phosphorylation of the IL-4Rα (Y575, Y603, Y633) enables the recruitment of signal transducer and activator of transcription 6 (STAT6) through the SH2 domain. Further STAT6 phosphorylation by JAK and dimerisation result in translocation to the nucleus, where it activates the transcription of IL-4 and IL-13 responsive genes. STAT6 dephosphorylation stimulates its export into cytosol, where it can be recruited into another cycle of tyrosine phosphorylation and nuclear entry [10].

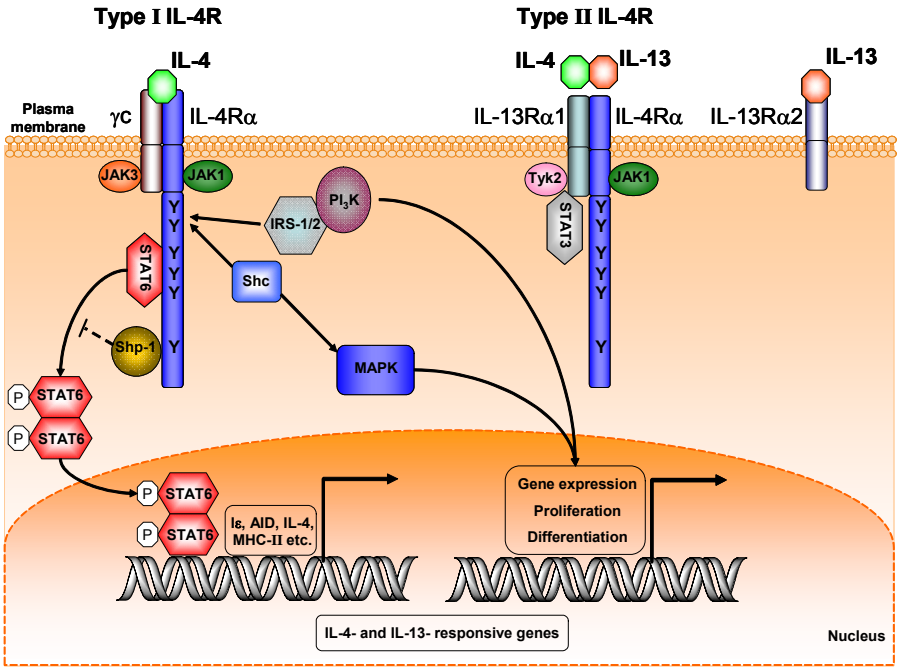

Figure 1: Illustration of IL-4R and IL-13R complexes focussed on IL-4Rα signalling [9,10]**.** Type I IL-4R complexes are composed of IL-4Rα and γc and bind exclusively to IL-4. Type II IL-4R complexes consist of IL-4Rα and IL-13Rα1 and bind to both IL-4 and IL-13. Signalling via IL-13Rα1 include additional intermediates like STAT3. The second IL-13 receptor IL-13Rα2 exclusively binds to IL-13. Signalling pathways activated via IL-4Rα are depicted in more detail. Receptor binding of IL-4 and IL-13 promotes activation of members of the JAK family that constitutively associate with component subunits of the receptor complexes. Thereby, several intracellular signalling cascades are initiated by phosphorylating specific tyrosine residues in the cytoplasmic domain of IL-4Rα. These phosphorylated residues act as connecting sites for signalling molecules. The first residue represents the insulin/interleukin-4 receptor (I4R) motif which facilitates the interaction with Shc and insulin receptor substrate (IRS) proteins. Phosphorylated IRS binds to phosphatidylinositol 3-kinase (PI_3-K) which controls several protein kinases regulating proliferation. Together with PI_3-K connected pathways, Shc mediates the regulatory functions of I4R motif and activates components of mitogen-activated protein kinase (MAPK) cascade. Phosphorylation of tyrosine residue cassette recruits transcription factor STAT6. JAK phosphorylate STAT6 which lead to its dimerisation and translocation to the nucleus, where it activates the transcription of IL-4 and IL-13 responsive genes like AID. The last tyrosine residue binds to the phosphatase Src homology domain 2 (SH2) containing inositol phosphatase (Shp-1), which is a negative regulator of receptor signalling. Thus, the multiple effector functions of IL-4Rα are transmitted by the STAT6 and the I4R pathways separately and in combination.

In addition to the JAK-STAT pathway, IL-4 also induces tyrosine phosphorylation of IRS-1 and/or IRS-2, which mediate the activation of PI_3-K. Thereby I4R motif, a conserved tyrosine residue of IL-4Rα (Y497, Y500), engages phosphotyrosine binding domain adaptor proteins, like IRS and Shc, which may be also involved in IgE regulation [9,11].

IL-4Rα also bears an immuno-tyrosine inhibitory motif (ITIM) site at Y713 and its adjoining residues, which binds to several regulatory phosphatases, including Shp-1. It is discussed as a negative regulator in IL-4Rα signalling in STAT6 activation [9].

CD40 is a member of the tumor necrosis factor receptors (TNFR) family (Figure 2). Since its cytoplasmic tail does not have an intrinsic kinase activity, downstream signalling is transmitted by the adapter molecules TNFR associated factors (TRAF) [12]. CD40 ligation results in rapid recruitment of TRAF1, TRAF2, TRAF3 and TRAF6 to the CD40 cytoplasmic domain [13]. TRAF2 and TRAF3 are drafted to the cell membrane after CD40 engagement, which allows the contact to membrane cholesterol-rich microdomains or lipid rafts. Subsequently TRAF2 induces its own as well as TRAF3 ubiquitylation. Degradation of these TRAF occurs in cytoplasmic proteasome [13].

TRAF1 is implicated in regulation of other TRAF and enhances the TRAF2 degradation. TRAF2 and TRAF6 are essential for c-jun kinase (JNK) and p38 activation, but also nuclear factor kappa B (NFκB) signal. TRAF3 negatively regulates JNK and NFκB activation by competitive binding of TRAF2 to CD40. Additionally, TRAF3 inhibits the interaction between B cell receptor and CD40 [13]. Besides TRAF numerous kinases, like germinal centre kinase (GCK) contribute to CD40 signal transduction [14]. The transcription factors activator protein 1 (AP1) is also induced and its signalling pathway involves JNK and p38 [13].

The activation of NFκB by TRAF2 and TRAF6 starts with NFκB inducing kinase (NIK). This MAPK phosphorylates and thus activates the inhibitor of NFκB (IκB) kinase complex (IKKα, IKKβ and IKKγ) which leads to phosphorylation of IκB proteins. Following IκB degradation, liberated NFκB translocates to the nucleus where it regulates the expression of a wide spectrum of genes [13,14,15].

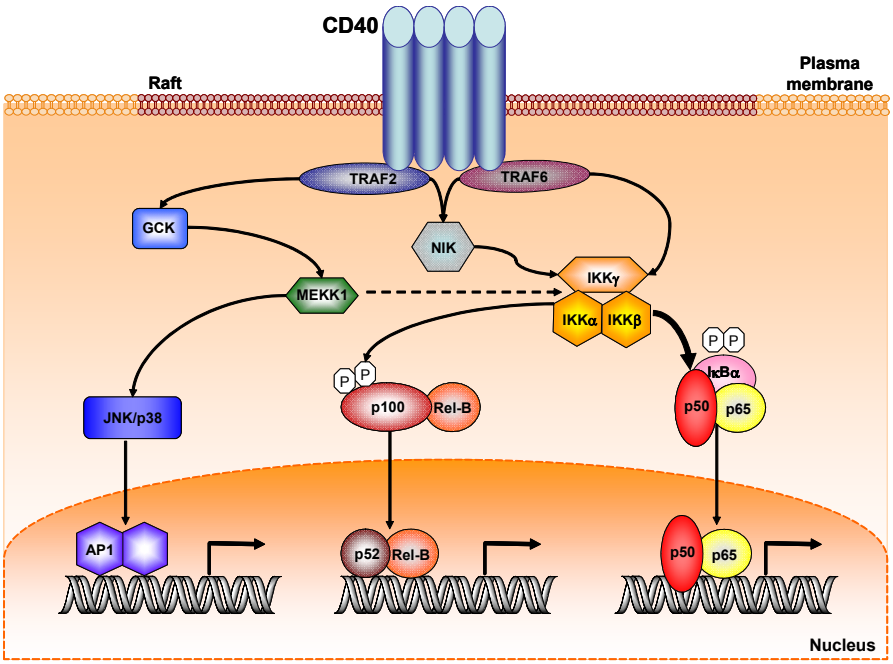

Figure 2: CD40–TRAF activated signalling cascades lead to gene regulation [13,16]. In B cells CD40 ligation activates NFκB pathway via TRAF2 and TRAF6. Thereby NFκB signalling is initiated through activity of NIK leading to activation of the IKK complex. IKK phosphorylates the inhibitory IκB thereby promoting their ubiquitylation and proteasomal degradation. Consequently, liberated p50/p65 heterodimers (NFκB type 1) translocate into nucleus regulating a wide spectrum of genes. Furthermore, IKK complex induces the formation of p52 through phosphorylation induced, ubiquitin dependent processing of p100. The subsequently activated p52/Rel-B heterodimers (NFκB type 2) target distinct elements. In B cells CD40 ligation mainly activates NFκB by the rapid NFκB type 1 pathway; beside this the more sustained NFκB type 2 cascade. TRAF6 additionally induces activation of the transcription factor AP1, whereas TRAF2 activates MEKK1 (MAPK/extracellular signal-regulated kinase [ERK] kinase kinase 1) and JNK.

Each immunoglobulin region is composed of a short germline exon (I), a repetitive GC-rich switch region (S) and several exons encoding the C_H region (Figure 3). In case of IgE switching, STAT6 and NFκB bind to epsilon germline gene promoter initiating sterile germline mRNA transcript (εGLT), which is not translated into a functional protein and lacks VDJ region. Both transcription factors synergise for IgE transcription and maximal IgE production [1,7].

STAT6 and NFκB also bind to a site in the upstream region of the AID gene and thereby contribute to an increase in available AID enzyme during the DNA rearrangement process [7].

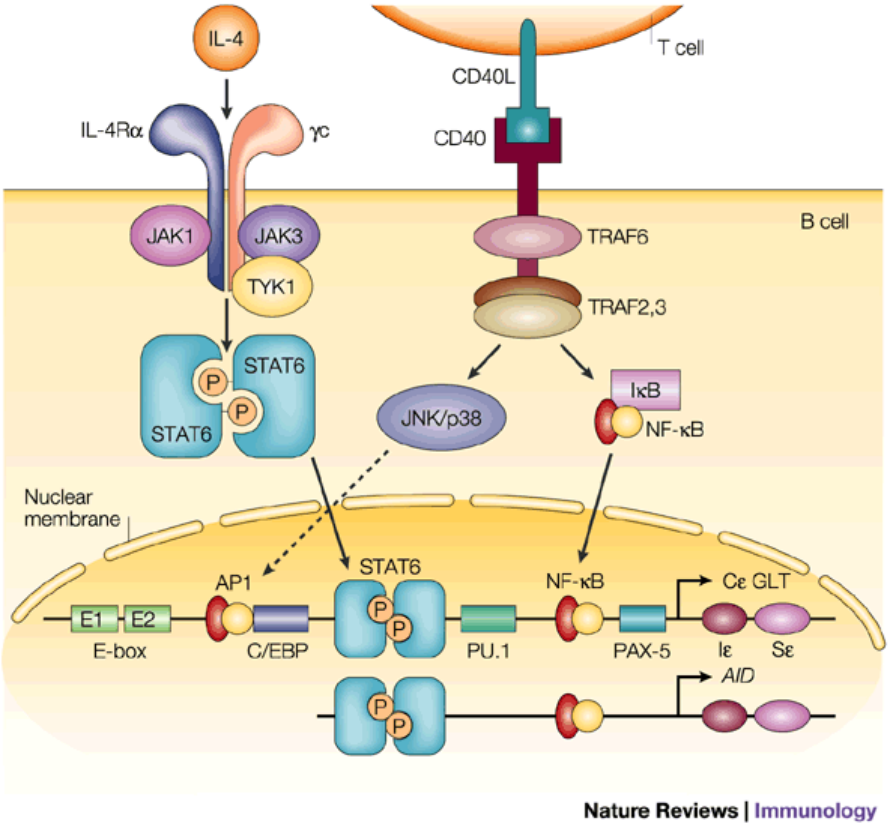

Figure 3: εGLT and AID transcription is induced by IL-4 and CD40 ligation [5]. Ligation of the IL-4R by IL-4 recruits and activates the tyrosine kinases JAK1 and JAK3, thereby activating STAT6. CD40 stimulation by CD40L on T cells permits the activation of TRAF leading to nuclear translocation of NFκB and AP1. IL-4R and CD40 signalling promote the synergistic enhancement of transcription of both the εGLT (Cε GLT) and AID by NFκB and STAT6. Transcription at the C_H locus, which is induced by specific cytokine signals, precedes CSR. εGLT originate at a promoter upstream of the I exon and do not encode functional proteins. C/EBP, CCAAT/enhancer binding protein; PAX5, paired box protein 5. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology (2003 Sep;3(9):721-32), copyright (2003); http://www.nature.com/nri/index.html.

The εGLT transcription is initiated at the Iε exon, transcribed through the S and C_H genes and completed at the usual termination sites for mature mRNAs (Figure 4). Association of εGLT with the genomic S region lead to formation of RNA-DNA hybrids (R-loops). The R-loop structure results in a single stranded DNA segment, which is targeted by AID. This enzyme desaminats dC to dU within S regions, thereby generating an U:G mismatch which is proc-

essed through uracil removal, base-excision or mismatch repair. In order to achieve CSR by an intrachromosomal deletion, double strand DNA breaks are brought into both the upstream Sμ/γ and the downstream Sε region [1,7,17]. Since there is an almost linear correlation between S region size and CSR frequency, the low IgE expression levels are supported by the small size of the Sε region [7].

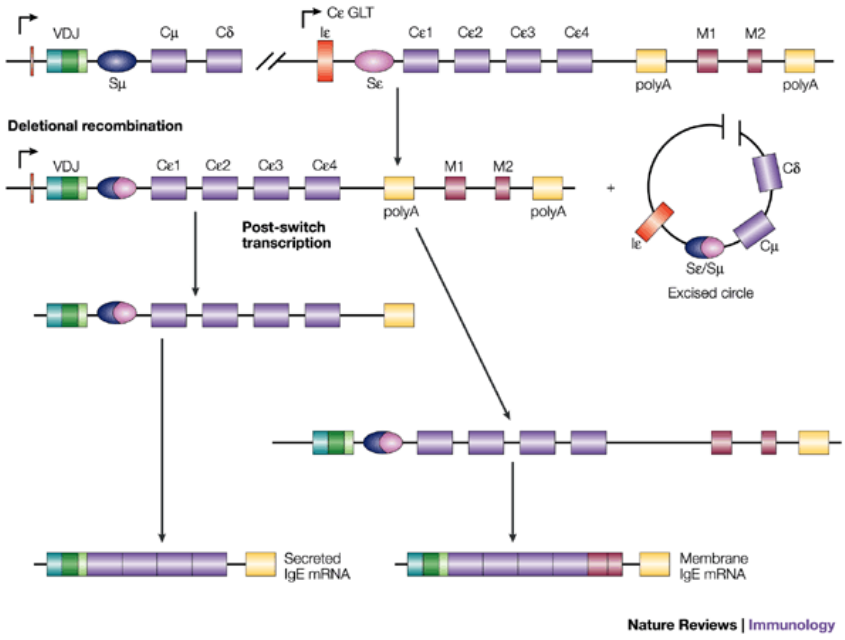

Figure 4: Deletional CSR of the human immunoglobulin locus [5]**.** During B cell development immunoglobulin heavy chain loci are rearranged and contain clusters of heavy chain variable (V), diversity (D) and joining (J) cassettes. This process results in a complete rearranged VDJ region encoding an antigen binding domain capable of generating intact μ and δ heavy chains. The production of other antibody isotypes that have the same antigen specificity requires an additional excision and repair process - known as deletional CSR. During CSR a defining variable region is molecularly linked with constant regions of different isotypes and distinct effector functions. This involves the excision of a piece of genomic DNA spanning from Sμ sequences to the targeted downstream S sequence. Association of the VDJ sequences to the Cε locus gives rise to an integral ε heavy chain gene and the production of intact IgE antibodies. Membrane IgE RNA is encoded by splice isoforms with the M1 and M2 exons. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology 2003 Sep;3(9):721-32), copyright (2003); http://www.nature.com/nri/index.html.

1.2 Atopic eczema

Atopic eczema is a chronically relapsing, inflammatory skin disease associated with erythema, severe pruritus, crusty and oozing plaques on forehead, face, neck and hands [18]. The pathogenesis is driven by a complex combination of environmental, genetic, immunologic factors and skin barrier dysfunctions [19,20].

The incidence of atopic eczema is increasing worldwide and varies in different populations. 10 to 20 % of infants and children and 1 to 3 % of adults are affected by this disease, whereas the prevalence of atopic eczema varies between 2 and 5 % in the whole population. In most cases, about 70 %, manifestation occurs within the first five years of life. At later age 30 to 60 % of children suffering from this disease develop respiratory diseases such as asthma or hay fever (atopic march) [20,21]. Thus, atopic eczema causes high costs and is a serious socioeconomic burden besides its negative impact on the quality of life of suffering patients [18,20,22,23].

Two types of atopic eczema have been delineated. Most patients (70 to 80 %) suffer from the extrinsic type of atopic eczema which is associated with an IgE mediated sensitisation. By contrast, the intrinsic type does not seem to be linked to IgE related mechanisms and affects 20 to 30 % of atopic eczema patients. Cutaneous lymphocyte associated antigen (CLA) expressing memory T cells are recruited to the skin where they produce higher amounts of T_H2 cytokines. IL-4 and IL-13 induce the isotype switching towards IgE, whereas IL-5 leads to eosinophil expansion and survival. Inheritance and other internal processes are associated here. Intrinsic atopic eczema is characterised by a lower IL-4 and IL-13 production compared to extrinsic form. However, few patients suffer from both extrinsic and intrinsic form [20,22].

1.2.1 Pathophysiology of atopic eczema

The pathophysiology of atopic eczema involves genetic predisposition, disturbed skin barrier function, defects in the antimicrobial immune defence and frequent allergic responses against allergens [19,20].

A high familial association has been described for atopic eczema. Genetic research was focussed on specific atopic eczema alleles, as well as identifying overlapping genes associated with other allergic disorders. Genetic polymorphisms have been assigned for a cluster of T_H2 cytokine genes located on chromosome 5q22-23, the IL-13 coding region and the IL-4Rα subunit (16q12) [20,21,24]. Heritable epidermal barrier defect has been shown to be connected to the filaggrin (filament-aggregating protein) gene (*FLG*). *FLG* mutations are a major risk factor for eczema associated asthma [21,24].

Atopic eczema is characterised by dry skin with increased transepidermal water loss. The disturbed barrier function results from a reduced activity of acid ceramidase and decreased ceramides content, the most important water retaining molecules in the extracellular space. Additionally, this dysfunction causes a reduction of sphingosine levels in the stratum corneum. Since sphingosines are potent antimicrobials, the colonisation with Staphylococcus aureus (S. aureus) is supported. Diminished levels of additional antimicrobial peptides, e.g. β-defensine 2 and cathelicidin, promote the ongoing colonisation of inflamed skin by various microorganisms [20,21].

The T_H2 predominance in atopic eczema patients, resulting from an increased frequency of allergen specific T_H2 cells and a decrease of interferon (IFN) γ producing cells, favours IgE production and peripheral eosinophilia. Disturbed cellular immunity, humoral factors like increased IgE synthesis and high production of related cytokines contribute to development of skin lesions [18,20].

Clinically unaffected skin in atopic eczema is characterised by sparse perivascular T cell infiltrate, increased number of T_H2 cells expressing IL-4 and IL-13, but not IFNγ [25] (Figure 5).

Initially, allergens penetrate into damaged skin and activate DC by cross linking FcεRI bound IgE molecules leading to an enhanced antigen presentation capacity. In this phase naive T cells are polarised into T_H2 cells producing associated cytokines. Additionally, MC are activated via antigen specific IgE and contribute to induction of the inflammatory response [22].

Very early events initiating atopic skin inflammation are not completely elucidated. Environmental allergens, scratching or microbial toxins cause skin injury thereby activating keratinocytes, MC and DC to release proinflammatory cytokines and chemokines, like IL-1, thymic stromal lymphopoietin (TSLP) and TNFα. Local proinflammatory mediator expression orchestrates adhesion to the endothelium and subsequent extravasation of cells and thus defining the nature of the inflammatory infiltrate. These mediators enhance the expression of adhesion molecules on vascular endothelium and facilitate the extravasation of inflammatory cells into the skin. In the tissue these cells respond to chemotactic gradients established by cytokines and chemokines originated from sites of injury or infection [20,26]. Cutaneous T cell attracting chemokine (CCL27) is pivotal in mediating the migration of CLA^+ T cells. Additionally, macrophage derived chemokine and activation regulated cytokine are increased in patients with atopic eczema. These molecules selectively recruit CCR4 expressing T_H2 cells. Dimension of thymus and activation regulated cytokine levels have been linked to severity of atopic eczema [20].

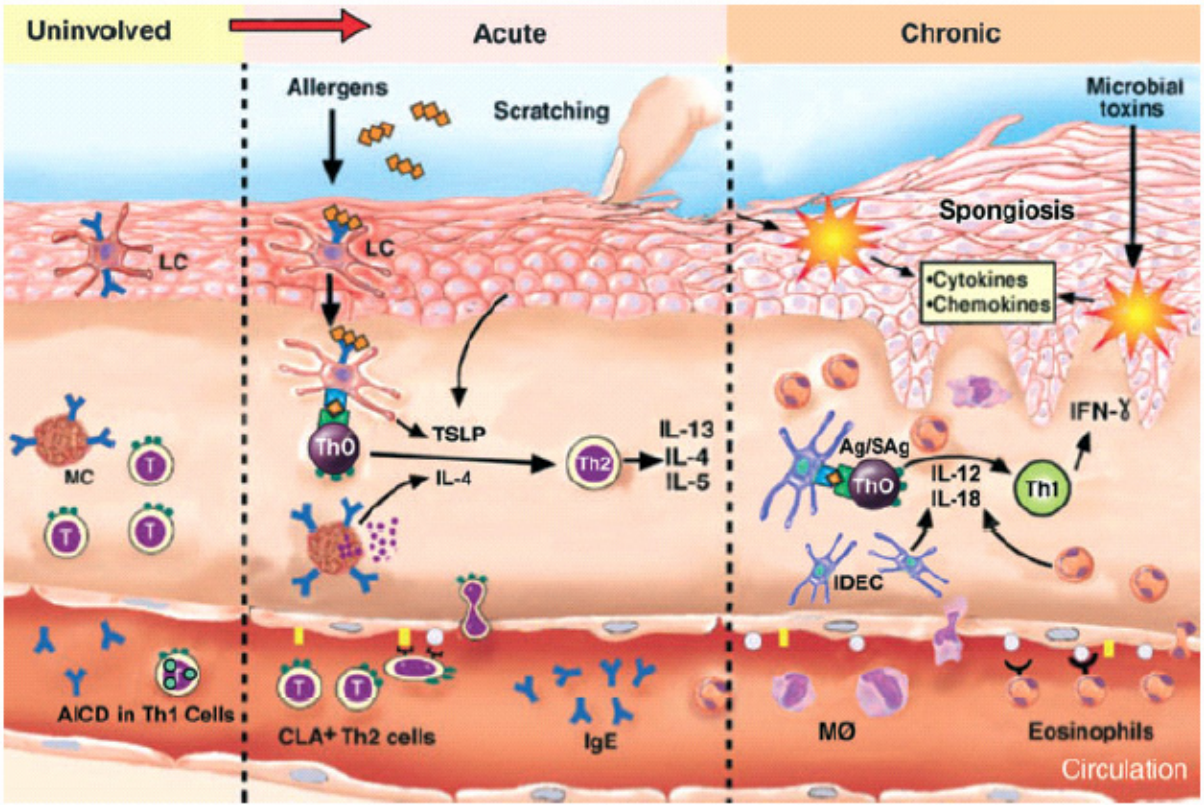

Figure 5: Immunological processes in the skin involved in the pathogenesis of atopic eczema [20]. Circulating CLA expressing T_H2 cells lead to elevated serum IgE and eosinophil numbers. These T cells recirculate through unaffected skin where they can engage allergen triggered IgE^+ LC and MC supporting T_H2 cell pattern. Skin injury provokes keratinocytes to release proinflammatory cytokines and chemokines thereby enhancing the expression of adhesion molecules on vascular endothelium and facilitating extravasation of inflammatory cells into skin. Keratinocyte derived TSLP and MC originated IL-4 enhance T_H2 cell differentiation. In acute skin lesions, T_H2 cell numbers are increased, but chronic eczema results in the infiltration of inflammatory dendritic epidermal cells (IDEC), macrophages (MΦ) and eosinophils. IL-12 production provokes the switch to a T_H1 cytokine milieu associated with increased IFNγ expression. Ag, antigen; SAg, superantigen; AICD, activation induced cell death. Reprinted from Akdis CA et al.: Diagnosis and treatment of atopic dermatitis in children and adults: European Academy of Allergology and Clinical Immunology/American Academy of Allergy, Asthma and Immunology/PRACTALL Consensus Report, J Allergy Clin Immunol 2006;118(1): 152-69, Copyright (2006), with permission from Elsevier.

Acute eczematous skin lesions are clinically designated by extremely pruritic, erythematous papules associated with excoriation and serious exudation. Memory $CD4^+$ cells infiltrate into the dermis. Compared to unaffected skin, acute skin lesions show a significantly larger num-

ber of T_H2 cells, but only few T_H1 cells. Importantly, LC, IDEC and macrophages in lesional and, to a lesser extent, in non-lesional skin bear IgE [20,25].

Due to ongoing inflammation, chronic lichenified skin lesions have undergone tissue remodelling. The clinical criteria are thickened plaques with increased markings (lichenification) and dry, fibrotic papules. Collagen deposition during chronic atopic eczema results from increased expression of the profibrotic cytokine, IL-11. While T cells remain present, although in smaller numbers than seen in acute atopic eczema, macrophages dominate the dermal mononuclear cell infiltrate. Increased number of IgE bearing LC and IDEC are found in the epidermis. Inflammatory response is also supported by eosinophils. The late phase switch to T_H1 depicts the chronicity of the atopic lesions, characterised by an increased production of the related cytokines, such as IFNγ and IL-18 [20,27].

1.2.2 Atopic eczema and fatty acids

Previous studies have shown abnormalities in fatty acid metabolism in atopic eczema. This aberration is hallmarked by an elevated concentration of the omega-6 (n-6) polyunsaturated fatty acid (PUFA) linoleic acid (LA, 18:2n-6) and reduced levels of LA metabolites in blood, milk and adipose tissue. The same phenomenon was observed for n-3 PUFA with an increased α-linolenic acid (ALA, 18:3n-3) concentration and a decrease of its metabolites. Interestingly, a positive correlation was found between LA concentration and IgE levels [28], but also an inverse association between γ-linoleic acid (GLA, 18:3n-6) or arachidonic acid (AA, 20:4n-6) levels and IgE [29]. This suggests a correlation of LA metabolism impairment and the risk of developing atopic eczema [28].

These aberrations in PUFA proportions reflect the reduced activity of the responsible enzyme Δ6-desaturase which may result from a enzyme mutation, altered expression, modifications in responsible cofactors, alterations in hormonal regulation or the presence of enzyme inhibitors [28]. This enzyme determinates the rate limiting step in desaturase / elongase pathway. The generated metabolites serve as precursors for immunomodulators such as PG and LT [30]. At birth these fatty acid alterations are already existent and precede the development of the skin lesions [28]. If there is a causal dependence, a deficit of certain essential fatty acids in infancy could impede normal skin function and lead to abnormal maturation and sensitisation of the immune system. Due to the memory of the immune system, these immunological aberrations and their relevance for the skin might not be resolved by correction of the fatty acid abnormalities after sensitisation. However, substitution of suitable fatty acids is expected to regulate the element of skin damage directly [28].

On this basis, several studies have been focused on counterbalancing this lack of n-6 PUFA with oral substitution of natural plant derived oils rich in GLA, such as primrose oil and borage oil, but a meta-analysis suggested no significant clinical benefit from n-6 PUFA in atopic eczema [30].

Because of its known antiinflammatory effects, some clinical trials were also focused on n-3 PUFA. Benefits by n-3 PUFA supplementation were shown for diseases like rheumatoid arthritis and psoriasis [31]. Subsequently n-3 PUFA application in atopic eczema patients was considered and examined clinically. However, only Mayser et al. [32] demonstrated a significant amelioration of the clinical outcome by daily infusions of n-3 PUFA. This therapeutical effect was accompanied by alterations in fatty acid profile of the membrane lipid pool as well as alterations in eicosanoid synthesis. Besides Gimenez-Arnau et al. [33] all other trials illustrated a non-significant advantage of n-3 PUFA by predominantly oral supplementation [34,35,36]. Few trials report additionally subjective patient ratings of disease expression with statistically significant results in some subscales in one study [37,38].

1.2.3 Murine models of atopic eczema

Animal models are indispensable research tools to study pathogenic mechanisms and to evaluate novel treatments. For this purpose an animal model has to meet distinct important criteria [39]:

- Display of the cardinal symptoms of the disease
- Reproducibility of induction / occurrence of disease phenotype
- Detailed knowledge of model organism
- Sufficient evolutionary homology
- Possible data transfer to man

Besides humans, mice, dogs, cats and horses have been reported to develop atopic eczema like skin lesions. A number of analogies regarding clinical manifestation, immunological aspects and histological findings between atopic eczema in humans and in dogs, cats or horses were determined. But only mice offer inbred strains displaying a stable disease phenotype.

The majority of the above mentioned criteria are fulfilled by most mouse models of atopic eczema and offer numerous additional advantages [39]:

- Small size
- Low housing costs
- Short generation time and large number of offspring
- Detailed knowledge about immune system and skin physiology
- Genetically modified mice (transgenic, conditional knock out, knock in, etc.)
- Control of age dependency and of environmental factors

Various mouse models are employed in the research field of atopic eczema. Genetically engineered mice, mice with spontaneous manifestation of atopic eczema like skin lesions, hapten induced models or humanised models of atopic eczema generated in mice with severe combined immunodeficiencies are applied for analysis of pathogenic mechanisms or evaluation of novel therapeutic concepts [39,40,41,42,43].

Epicutaneous (e.c.) exposure to allergens is an important trigger factor in pathogenesis of atopic eczema [22]. Different protein sensitisation protocols have been used to induce atopic eczema like skin lesions in mice, thereby varying the paths of antigen application for sensitisation, using different mouse strains, applying various proteins, allergen extracts or recombinant allergens. Each of these protocols was designed to address particular aspects of atopic eczema [39]. Adapted from this, Wang et al. [44] and Spergel et al. [45] established a murine model of atopic eczema elicited by repeated e.c. exposures to ovalbumin (OVA). This model features significant increased total and antigen specific IgE, significant epidermal thickening, cellular infiltration composed of T cells, eosinophils and macrophages as well as a local upregulation of IL-4, IL-5 and INFγ mRNA. Thus, several classical hallmarks of human atopic eczema are displayed by this model. E. c. sensitisation with protein is a potent inducer of systemic T_H2 response. [46].

However, these models describe important aspects, but up to now no model has integrated all aspects of the complex pathophysiology of atopic eczema. One important limitation is the difficulty of extrapolation from animal studies to human skin, due to the existing differences in immunological as well as in skin (barrier) parameters [47].

1.3 Polyunsaturated fatty acids (PUFA)

Historically “fat” has been considered important as a high calorific nutrient and a source of essential fatty acids. Today, fat and in particular fatty acids have been accepted as important biological regulators. Modifications in dietary fatty acids change cell membrane structure and function. Furthermore, fatty acids differentially influence the production of cytokines, chemotaxis and other immunologic factors. They are known to modulate signal transduction, enzyme activities, receptor expression, cell proliferation and differentiation [48].

1.3.1 Nomenclature and molecular structure of PUFA

One criterion for the classification of fatty acids is the number of carbon atoms present in hydrophobic hydrocarbon chain (chain length). Thus it is distinguished between short chained (< 4), middle chained (6 - 10) and long chained fatty acids (> 10). Besides saturated fatty acids, without any double bond and monounsaturated fatty acids, containing a single double bond, there are PUFA which comprise at least two double bonds in their hydrocarbon chain. In line with the nomenclature, PUFA are designated by their chain length (C), the number of double bonds (D) and the position of the first double bond proximal to the terminal methyl group (n-). Therefore, the structure of fatty acids is described by the commonly used short hand nomenclature as following C:Dn-x.

According to their fatty acid precursor, four independent PUFA families are classified: The omega-3 (n-3) series are derived from ALA (18:3n-3), the n-6 series descended from LA (18:2n-6); the n-9 from oleic acid (OA, 18:1n-9) and the n-7 series from palmitoleic acid (16:1n-7). It is important to mention that beyond PUFA only LA and ALA are essential, i.e. they cannot be synthesised in the human body and thus have to be obtained from the diet [49,50,51].

1.3.2 Dietary Sources of PUFA

LA is mainly found in cereals, eggs, vegetable oils, like sunflower and corn oils, margarine and whole grain breads. The main dietary sources of ALA are vegetable oils like canola oil, flaxseed oil, linseed and rapeseed oils, but also walnuts.

The longer fatty acids of n-3 and n-6 PUFA are either biosynthesised after intake of their precursors or are obtained directly from animal and marine sources. AA is found in modest amounts in human milk and in small amounts in cow’s milk. Egg yolk, meat, but also liver and brain contain large amounts of AA.

The major dietary source of eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) is marine oily fish and some species of algae and other seafood. Thereby the content of EPA and DHA varies from species to species according to diet, water temperature and season. Fresh water fish is unlikely to contain significant amounts of EPA and DHA [52].

1.3.3 Physiological function of PUFA

PUFA and fatty acids in general exert a variety of physiological functions within immune cells. They operate as [53]:

- Energy sources
- Elements of cell phospholipids contributing to the physical and functional membrane characteristics
- Covalent modulators of protein structure modifying their location and function
- Regulators of gene expression either through effects on receptor activity, on intracellular signalling processes or on activation of transcription factors
- Precursors for bioactive lipid mediators like PG, LT, lipoxins and resolvins

1.3.4 Metabolism of PUFA

In most tissues the metabolism of PUFA includes the alternating sequence of ∆6-desaturation, elongation and ∆5-desaturation (Figure 6). A new cis-double bond is formed by removal of two hydrogen atoms followed by insertion of two carbon atoms from glucose metabolism in order to elongate the fatty acid chain. Thereby ∆6-desaturase catalyses the conversion of LA (18:2n-6) to GLA (18:3n-6) whereas ∆5-desaturase is responsible for the generation of AA (20:4n-6) from dihomo-gamma linolenic acid (DGLA, 20:3n-6). The intermediate step, the transformation of GLA to DGLA, is catalysed by the elongase. The same set of Δ6- and Δ5-desaturases and elongases metabolise ALA, LA and OA with the following sequence of preference: n-3 > n-6 > n-9 [49,54,55].

Generated longer PUFA are incorporated into cell membrane. After liberation by phospholipase (PL) they serve as precursors of bioactive lipids like eicosanoids, protectins and resolvins [31]. The immune cell phospholipids from typical Western diet consuming individual contain about 20 % of fatty acids as AA, 1 % EPA and 2.5 % DHA. An altered consumption of certain fatty acids can modify this composition [53]. Thus, increased (long chain) n-3 PUFA intake leads to higher proportions of EPA and DHA in cell phospholipids. This incorporation occurs dose-dependently and is partly at expense of AA [31,50,54].

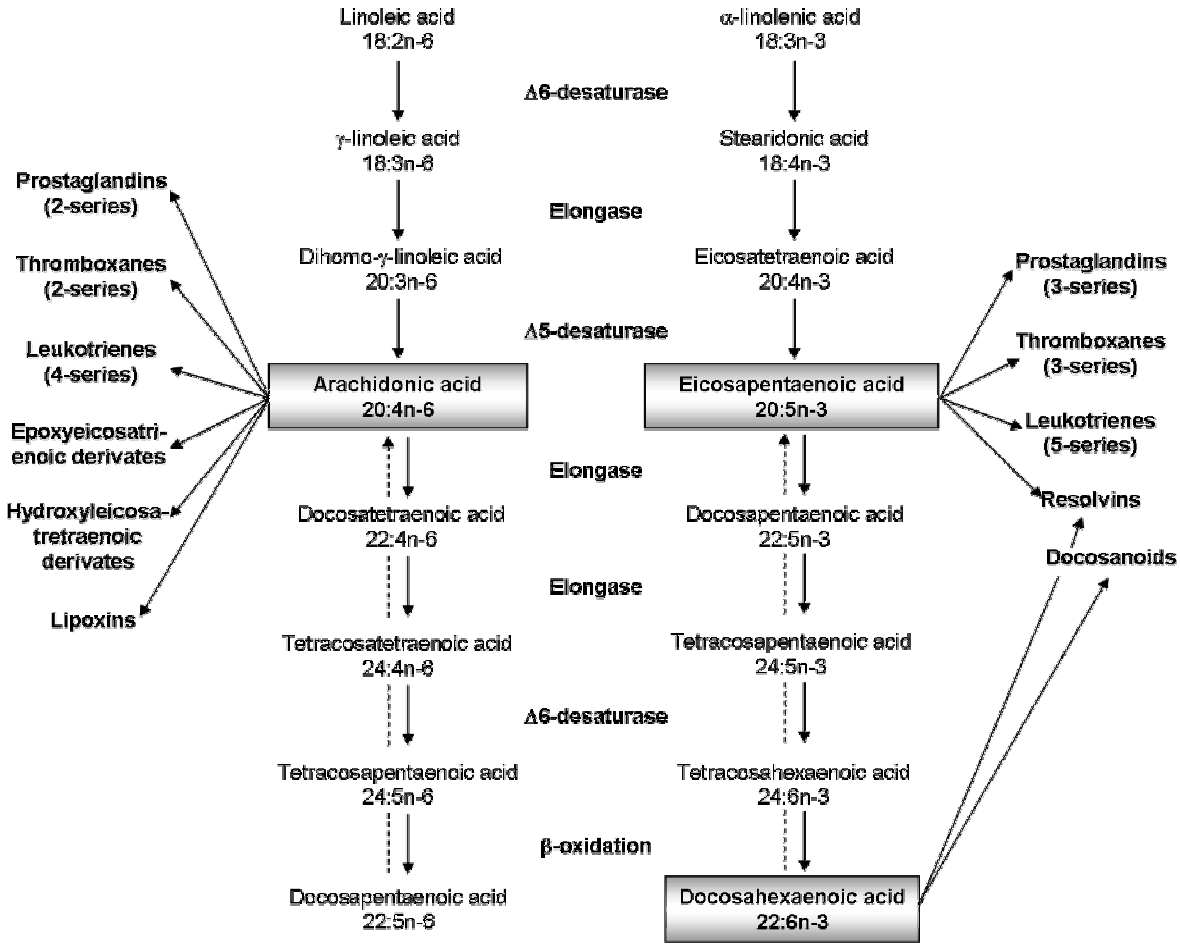

Figure 6: Metabolic pathways of n-6 and n-3 long chain PUFA, according to [54,56]. Broken arrows represent retroconversion cascades.

1.3.5 PUFA and immune system

On the basis of their physiological functions, fatty acids can mediate their biological action at different cellular levels [53]:

- By alterations in the pattern of lipid mediators
- Through changes in the physical membrane characteristics
- By modulation of membrane receptor expression, activity or avidity
- By alteration of intracellular signal transduction

One key link between PUFA action and function of the immune system is the generation of eicosanoids and resolvins from 20-carbon PUFA. Cell membrane phospholipids contain large

amounts of AA. This n-6 PUFA is the major precursor for eicosanoids, a family of hydroxylated PUFA with a wide range of functions on inflammatory and immune responses [31,53].

Cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P 450 pathways competitively metabolise AA and EPA to eicosanoids such as PG, thromboxanes (TX), LT, lipoxins and epoxy-compounds. Incorporation of increased amounts of EPA and DHA into cell membranes results in a higher formation of EPA derived products at expense of AA derived mediators. This is caused by decreased availability of AA as enzyme substrate and inhibition of AA metabolism [53,57]. EPA and DHA are poor COX and LOX substrates compared to AA. Due to a mislocalisation of carbon 13, EPA is only inadequately metabolised by COX-1. Therefore, EPA competitively inhibits AA metabolism to its eicosanoids. Equally, DHA inhibits AA derived mediator synthesis either itself or by retroconversion to EPA [58].

After liberation from membrane phospholipids the typically highly abundant AA is metabolised through COX to PG and TX of the 2-series, e.g. PGE_2 and PGF_{2a}. Metabolism of AA via 5-LOX provides 5-hydroxyeicosatetraenoic acid and the 4-series of LT, e.g. LTA_4 and LTB_4 [58]. These mediators have been shown to exert proinflammatory but also antiinflammatory properties [54].

Since EPA also operates as a substrate for COX and LOX, an increased availability of EPA potentially results in increased production of EPA derived eicosanoids such as PGE_3 and 5-series LT [53]. The functional significance of these mediators originated from EPA is the proven lower biological activity compared to those from AA. For example, LTB_5 is 10- to 100-fold less potent neutrophil chemotactic agent than LTB_4 and PGE_3 [31]. Long chained n-3 PUFA are also metabolised to resolvins (from EPA and DHA) and docosanoids (from DHA) through pathways involving COX and LOX. These mediators are antiinflammatory, immunomodulatory and resolve inflammation [53,54,59]. However, the physiological or pathophysiological outcome is designated by cells present, the nature of stimulus, timing of eicosanoid generation, concentrations of different bioactive lipids as well as sensitivity of target cells and tissues to generated mediators [53].

N-3 PUFA are mainly found at position sn-2 of membrane phospholipids, thereby replacing AA. PUFA incorporation into membrane lipids decreases the membrane microviscosity influencing the mobility, expression and function of membrane proteins [54]. Importantly, n-3 PUFA containing phospholipids are also found in lipid rafts, thereby modulating cellular signalling processes [49,58]. “Membrane rafts are small (10 to 200 nm), heterogeneous, highly dynamic, sterol and sphingolipid enriched domains that compartmentalise cellular processes. Small rafts can sometimes be stabilised to form larger platforms through protein–

protein and protein–lipid interactions" [60]. Proteins are anchored in the membrane rafts exoplasmically by glycosyl phosphatidylinositol or cytoplasmically by acyl moieties. Many proteins involved in signal transduction are modified in this way and / or concentrated in lipid rafts [58].

PUFA treatment was shown to modify the cytoplasmic leaflet of lipid rafts, thereby selectively displacing acylated proteins and integrating n-3 PUFA. Moreover, dietary n-3 PUFA supplementation reduces significantly the sphingomyelin content of lipid rafts in vivo. This causes an altered exoplasmic membrane leaflet and folds acyl chains in the cytoplasmic leaflet. Incorporation of PUFA as a replacement for saturated fatty acids or changes in raft lipid composition could lead to modified protein acylation and thus protein displacement from lipid rafts [58]. Additionally, changing the fatty acid composition of immune cells affects phagocytosis, T cell signalling and antigen presentation at the membrane level suggesting important roles of fatty acids in membrane order, lipid raft structure and function as well as membrane trafficking [53].

Cells are responsive to extracellular signals by an up or downregulated expression of specific genes which leads to altered metabolism, proliferation, differentiation or apoptosis. Receptor mediated signal transduction pathways transmit these extracellular signals to their intracellular targets. In addition to their roles as structural components of membrane lipids and as precursors of eicosanoids, fatty acids can act as second messengers or regulators of signal transducing molecules [61]. Signalling molecules that may be modulated by different fatty acids are divided into three groups:

1.3.5.1 Signalling molecules that require fatty acid acylation for membrane translocation and functional activation

Translocation and protein function can be dramatically influenced by covalent attachment of long chain fatty acids to a wide range of proteins. Many molecules involved in transmitting extracellular signals are acylated for their membrane translocation. Cotranslational myristoylation and posttranslational palmitoylation are described in this manner [61].

1.3.5.2 Lipid mediators containing different fatty acids or free fatty acids

PI_3-K phosphorylate various phosphoinositides and regulate multiple cell functions such as chemotaxis and apoptosis. The activity of the newly generated phosphatidylinositol 3,4,5-trisphosphate varies with types of fatty acids in the *sn*-1 and *sn*-2 positions of phosphoinositides which can be altered by dietary fatty acids [61].

Hydrolysis of sphingomyelin creates ceramide containing one fatty acyl moiety linked to the sphingosine backbone by an amide bond. Ceramide acts as an intracellular signal effector molecule with several downstream targets onto various extracellular signals. Thus, controlling ceramide activity by dietary fatty acids would exert profound nutritional implications [61].

Since the composition of membrane phospholipids is influenced by diverse dietary fatty acids, fatty acyl residues of diacylglycerol (DAG) may also be altered by dietary fatty acids. It has been shown that the capacity to activate the protein kinase C (PKC) is regulated by fatty acid esterification at the sn-1 or sn-2 position of DAG [61]. The activation of PKC is enhanced via DAG by *cis*-unsaturated fatty acids like DHA, whereas other PUFA, especially AA increases the activation of PLCγ. Additionally, AA and other PUFA control the activities of multiple cellular proteins, including ion channels and protein kinases [61].

1.3.5.3 Signalling molecules that can be modulated by different fatty acids

Nuclear receptors, a family of ligand activated transcription factors, are able to control several genes involved in lipid metabolism and inflammatory signalling directly and indirectly. Ligand binding leads to conformational changes which enable the nuclear receptors to dissociate their corepressors and recruit coactivator proteins to allow transcriptional activation [54]. Various PUFA and prostanoids are ligands for peroxisome proliferator activated receptor (PPAR), a nuclear hormone receptor regulating the transcription of genes involved in lipid metabolism but also in diverse cellular responses [61]. Longer chain PUFA, but not shorter chain monounsaturated or saturated fatty acids, are able to repress the liver X receptor (LXR) enhancer complex in the sterol regulatory element binding protein (SREBP) 1c promoter region [48]. The discussed “cross-talk” between PPAR signalling, retinoic acid X receptor (RXR), SREBP expression and LXR may influence basically cell lipid homeostasis in a highly complex but coordinated manner. Due to the various characteristics and the exhibited transcriptional regulatory properties of each n-3 PUFA, the subsequent effects of these interactions are also highly complex [48,54].

Furthermore, it has been shown that n-3 PUFA inhibit NFκB activity directly. EPA diminishes degradation of the IκB thereby blocking NFκB. AA derived prostanoids inhibit NFκB translocation and activation by potently blocking the IKK complex and thus retaining NFκB in the cytoplasm. Consequently, n-3 PUFA and fatty acid derived mediators modulate manifold the NFκB pathway [54].

1.3.6 Docosahexaenoic acid (DHA)

Due to the structural characteristics of 22 carbons and six double bonds, DHA (22:6n-3, Figure 7) represents an extreme example of n-3 PUFA family. Primarily dietary studies revealed the favourable impact of DHA on diseases like rheumatoid arthritis, asthma, dermatitis and psoriasis [62,63]. Thus, the cell membrane is discussed to be one major target of DHA action. DHA is readily incorporated into membrane phospholipids and has been demonstrated to alter significantly many basic properties of membranes including acyl chain order and phase behaviour, permeability, flip-flop and protein activity thereby modulating the local structure and function of cell membranes [54,62,64]. Although DHA is normally stored in membranes as the fatty acid portion of certain phospholipids, free DHA has been also suggested to possess transcellular activity [65].

Various effects by DHA supplementation may be also due to a shift in eicosanoid formation. Thus, DHA is known to reduce PGE_2 production [66] but also the expression of the PG synthesising enzyme COX-2 [67,68]. Additionally, DHA enhances, probably in combination with EPA, the levels of COX and LOX driven mediators, e.g. resolvins, docosatrienes and protectins. These lipid mediators have been shown to act antiinflammatorily [69,70]. The mechanisms of such downstream events are complex and the antiinflammatory properties of DHA cannot be fully explained by a simple exchange of eicosanoid families [71].

Besides this, DHA is discussed to act via nuclear receptors, as it has been shown that DHA is e.g. a PPAR activator [54,72] as well as RXR ligand [73].

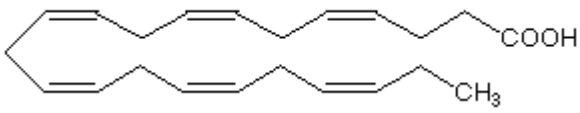

Figure 7: Structure of DHA

1.4 Objective

Due to the increased incidence and limited causal therapeutics allergic diseases have become a major medical issue in the Western world [1]. The reasons of this development are not completely understood. In recent years research has pointed to the possible role of environmental factors like changing Western dietary habits. The increased intake of n-6 PUFA (margarine, vegetable oil) and decreased n-3 PUFA (oily fish) consumption have been hypothesised to contribute to this development. However, the associations with n-6 and n-3 PUFA are complex and may differ between different allergic diseases [74,75].

Although many immunomodulatory properties of DHA are described, the underlying mechanisms of DHA action are not exactly understood. Therefore, the following objectives were investigated:

- First, the target cells of antiallergic DHA action were identified and subsequently its molecular mechanisms investigated.
- Within a randomised clinical study the therapeutical efficacy of high-dose DHA supplementation was examined in patients with extrinsic atopic eczema. Beside the clinical effects, immunological parameters were analysed.
- Finally, the clinical efficiency of oral DHA administration was verified in a mouse model of protein mediated eczema and the underlying local processes in eczematous skin examined in more detail.

2 Materials and methods

2.1 Materials

Equipment, software, commodities, chemicals, antibodies and buffer and solutions used are listed in detail in the appendix (page 94 - 105).

2.2 Methods

2.2.1 Donors and cells

The in vitro experiments were approved by the ethical committee of the Charité–Universitätsmedizin Berlin. Therefore, blood was obtained from allergic patients and healthy persons.

Atopic donors were patients suffering from rhinoconjunctivitis allergica, asthma allergica and from atopic eczema defined according the criteria of Hanifin and Rajka [76] with serum IgE concentrations below 2500 ng/mL. Donors with allergic conjunctivitis, allergic rhinitis and asthma were recruited based on their history and positive prick testing as well as patients with atopic eczema from the Department of Dermatology and Allergy, Charité.

None of the enrolled donors received a systemic immunosuppressive medication during the past three months before the blood sample was taken. Usage of topical steroids (class II-III) or antihistamines was not excluded.

Blood samples from age and sex matched healthy volunteers (no type I allergies or positive family history of atopic diseases) and blood filters from blood donation of the Charité with IgE levels up to 100 ng/mL were chosen as control.

2.2.2 Participants and clinical study

The clinical study was approved by the ethical committee of the Charité–Universitätsmedizin Berlin and was performed in the Department of Dermatology and Allergy, Charité from January 2005 to June 2005. Written informed consent was obtained from each participant and eligibility was confirmed by a dermatologist.

Patients suffering from atopic eczema aged between 18 and 40 years were enrolled into the study. Atopic eczema was defined according the criteria of Hanifin and Rajka [76]. Exclusion criteria were pregnancy, lactation, seafood allergies and consumption of dietary supplements, systemic immunomodulatory or immunosuppressive therapy in the last three months, presence of other systemic and chronic disorders than allergic diseases. Participants were al-

lowed to use standard therapy for atopic eczema, including emollients, topical corticosteroids and oral antihistamines. Subjects were asked to maintain their habitual diet and activities throughout the intervention. Randomisation was stratified according to gender, age (cut off 29 years) and body mass index (BMI; cut off 25 kg/m^2) in a ratio of 1:1 using a randomised allocation schedule (based on block randomisation). Allocation concealment was assured by a sealed envelope up to finishing the data analysis. Recruited participants were randomly allocated to receive a treatment with either DHA capsules (n = 21) or control supplement (n = 23) by an independent clinician.

Variable	Control (n = 23)	DHA (n = 21)
Sex: female/male	16/7	14/7
Age [years]:	26.7 [19.2 – 37.1]	26.5 [18.5 – 39.8]
Height [cm]:	172.5 ± 8.5	173.8 ± 10.6
Weight [kg]:	67.8 ± 13.3	72.5 ± 17.8
BMI [kg/m^2]:	22.7 ± 4.0	24.2 ± 5.2
SCORAD:	35.4 [17.2 – 63.0]	37.0 [17.9 – 48.0]
Total IgE [ng/mL]	983.9 ± 976.4	1336.1 ± 2135.0
intrinsic/extrinsic type	4/19	6/15

Table 1: Baseline characteristics of study population.

Each participant was instructed to consume seven capsules daily (with main meals) for 8 weeks. Every capsule contained 1 g of the respective oil. From DHA capsules, subjects consumed 5.35 g/d DHA and 0.37 g/d EPA in form of ethyl esters and control capsules provided 4.17 g/d caprylic acid (C8:0) and 2.84 g/d capric acid (C10:0). Participants were clinically assessed at baseline (week 0), week 4 and 8 as well as 12 weeks after finishing the supplementation period (week 20) (Figure 8). The severity of atopic eczema was determined by Severity Scoring of Atopic Dermatitis (SCORAD) score through the same physician. At the first visit a full medical history was taken, physical examination was carried out and capsules were delivered. At every time point immunological parameters, i.e. anti-CD40/IL-4 stimulated IgE production, activation status of B cells and monocytes as well as cytokine production in peripheral blood mononuclear cells (PBMC) were analysed in our laboratories. Additionally, clinical safety was measured by laboratory parameters (liver enzymes, complete blood count and serum lipids).

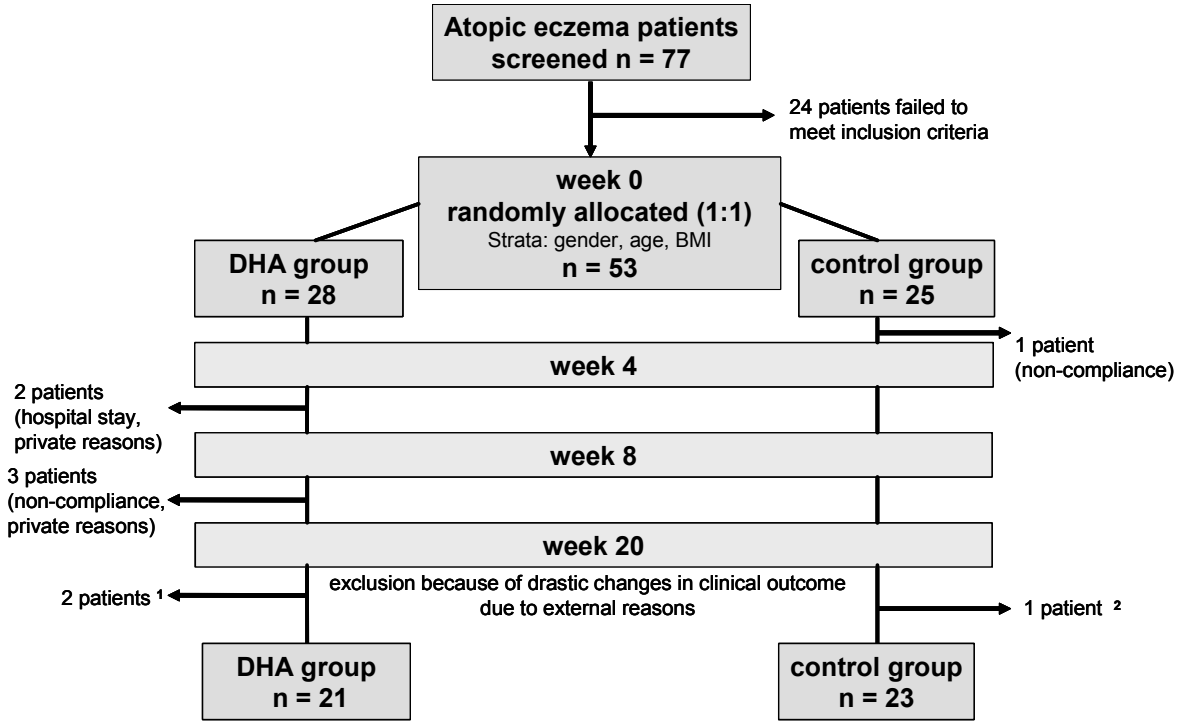

Figure 8: Study flow chart. Exclusion because of [1] unwanted interruption of the allowed standard medication leading to a drastic aggravation and [2] excessive sunbathing during holidays.

2.2.3 Mouse model of allergen induced eczema

Female BALB/c mice (8 to 10 weeks) were purchased from the Federal Institute of Risk Assessment, Berlin, Germany. All mice were kept within an accredited animal facility at the Charité-Universitätsmedizin Berlin and maintained under specific pathogen free conditions at controlled room temperature (21 ± 1°C), constant relative humidity (55 ± 5 %) and 12 hour light / dark cycle. Procedures were performed in compliance with protocols approved by the local State Office of Health and Social Affairs.

As shown in Figure 9, mice were sensitised intraperitoneally (i.p.) on days 1 and 14 with 100 µl of 10 µg OVA adsorbed to 1.5 mg $Al(OH)_3$ or injecting phosphate buffered saline (PBS) as control. E.c. OVA application was performed by using patch test method as previously described [45]. Briefly, mice were anaesthetised with an i.p. injection of Ketamin / Rompun. The dose was adapted according to body weight and application rate. Each mouse was weighed and the appropriate volume was calculated. Afterwards, the belly skin was shaved and 100 µg OVA adsorbed to 1.5 mg $Al(OH)_3$ or PBS was applied twice a week by patch test method. Each mouse had a total of three one-week exposures to the same skin site separated by two-week intervals without any application. For treatment evaluation non-

sensitised mice were used as negative control and PBS patched mice were referred as patch control.

On day 64 the phenotypical outcome was evaluated, animals were sacrificed by cervical dislocation and samples were taken for further analysis. Skin biopsies were frozen by liquid nitrogen. Blood samples were centrifuged at 3200 g for 10 min at 4°C. Serum was stored at -80°C.

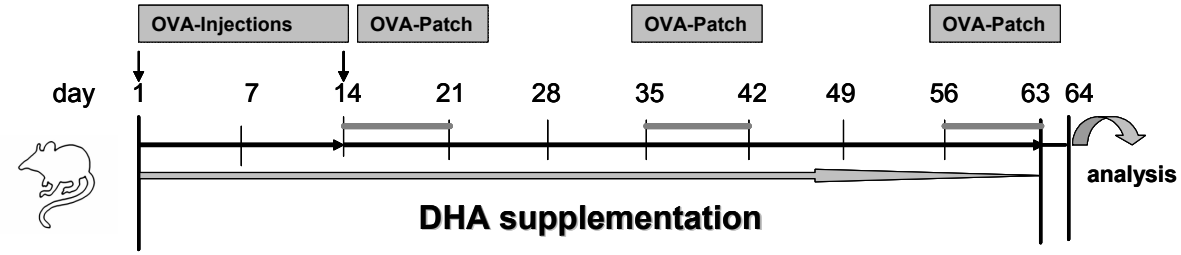

Figure 9: Sensitisation schedule for protein induced dermatitis and oral DHA application in mice. Mice were sensitised i.p. with 10 µg OVA / alum at days 1 and 14 (black arrows) and by e.c. patches with 100 µg OVA / alum (grey lines) with a total of three one-week exposures to the same skin site separated by two-week intervals without any application. Dietary DHA was applied during the whole experimental period. The e.c. sensitisation comprised three one-week exposures. On day 64 mice were sacrificed and samples were collected.

2.2.3.1 Diet

During sensitisation mice were treated with standard solid food for rats and mice (Ssniff®Spezialdiäten, Soest, Germany) containing 2 % or 4 % DHA. Admixture of fatty acid blend, pellet forming and packing were performed by Ssniff® under cold and air-tight conditions. Therefore, 4 % of DHA500TG and 5.7 % of DHA700EE was added to standard solid food to adjust 2 % and 4 % DHA in diet, respectively (Table 2). The basic food of control and experimental diet was originated from the same charge. The different diets were stored dark and protected from oxygen. Food and water were administered ad libidum. Nevertheless consumption was monitored.

Treatment group Fatty acid	Control Composition [%]	2 % DHA Composition [%]	4 % DHA Composition [%]
C14:0	0.3	n.d.	n.d.
C16:0	1.5	n.d.	n.d.
C16:1	0.3	n.d.	n.d.
C18:0	2.5	3.7	0.1
C18:1	19.1	6.9	1.4
C18:2	5.5	1.6	0.2
C18:3 n-3	7.1	0.2	0.1
C18:3 n-6	0.3	0.5	n.d.
C18:4 n-3	0.6	0.3	0.1
C20:4 n-6	n.d.	2.8	1.8
C20:4 n-3	n.d.	0.7	0.5
C20:5 n-3 (EPA)	n.d.	8.3	5.6
C22:5 n-3	n.d.	3.1	3.5
C22:6 n-3 (DHA)	n.d.	57.5	76.4

Table 2: Fatty acid composition of lipids in experimental diet; n.d. - not detectable.

2.2.3.2 Clinical Evaluation

Evaluation of severity of OVA induced eczematous skin lesions was performed by a skin scoring system, which considers typical skin features of human atopic eczema, erythema, edema / papulation, excoriation / crusting, dryness and extension [76,77]. The total clinical skin score (CSS) was initially described in a murine model with spontaneous manifestation of atopic eczema like skin lesions [78]. For calculating total CSS, parameters were graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) by two independent observers. The total score was taken as index of dermatitis severity ranging from 0 (no skin lesion) to 15 (severe skin lesion) (Figure 10).

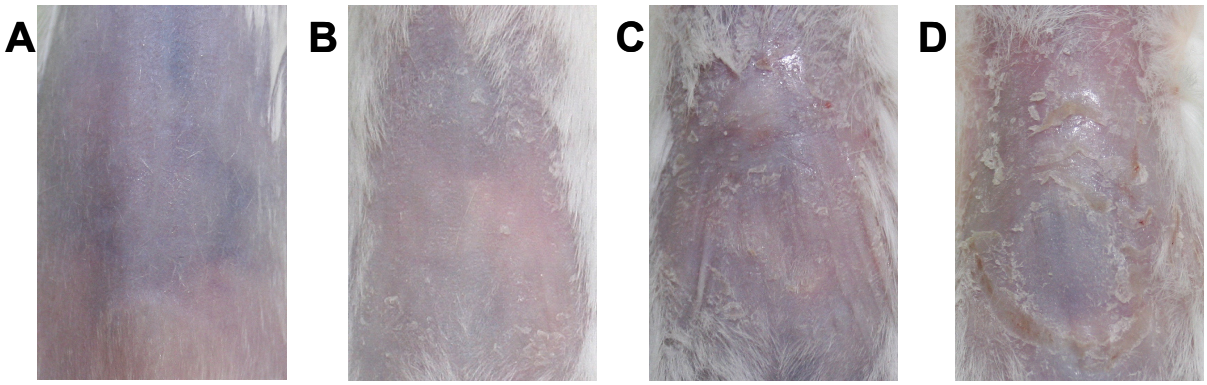

Figure 10: Illustration of total CSS according to clinical outcome: A) None, **B)** mild, **C)** moderate and **D)** severe protein induced eczema. For calculating total CSS, clinical parameters (erythema, edema / papulation, excoriation / crusting, dryness and extension) were graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) by two independent observers. The total score was taken as index of dermatitis severity ranging from 0 (no skin lesion) to 15 (severe skin lesion).

2.2.4 Cell culture methods

To ensure sterility, all cell isolation and culture procedures were performed under flow bench and with sterile / autoclaved commodities and reagents.

2.2.4.1 Cell isolation methods

Human PBMC were isolated by density gradient centrifugation with ficoll hypaque isolation (d = 1.077 g/mL) at 300 g, 20 min, room temperature.

B cells were purified by magnetic cell sorting (MACS) using anti-CD19-coupled magnetic beads. Briefly, 2×10^8 PBMC were incubated in 300 µL MACS buffer and 100 µL autologous serum for 1 min, followed by 12 min of incubation with 100 µL beads at 4°C. $CD19^+$ B cells were separated by magnetic double positive selection with the autoMACS. The purified cell population contained more than 99% B cells as assessed by flow cytometry. In previous studies, this procedure has been shown not to activate B cells [79]. Cell count measurements of the single cell suspensions were performed with CASY® cell counter.

2.2.4.2 Cell culture conditions

All cell cultures were carried out at 37°C in humidified air and 5 % CO_2 atmosphere. The culture medium RPMI1640 was supplemented with L-glutamine (2 µM), penicillin (100 U/mL), streptomycin (100 µg/mL) and 10 % heat inactivated fetal calf serum (FCS), with the exception of analysis of STAT6 phosphorylation and NFκB translocation which was performed in

pure RPMI1640. DHA, dissolved in 100 % ethanol, was portioned and stored at -20°C for a maximum of six months. The vehicle control was performed in culture media containing the same volume of ethanol (0.001 %). Toxic effects of DHA were excluded by propidium iodide and trypan blue staining.

2.2.4.3 Carboxy-fluorescein diacetate, succinimidyl ester (CFSE) labelling

B cells were suspended in PBS containing 5 µM CFSE for 5 min at room temperature and subsequently washed. Cells were stimulated with anti-CD40 (1 µg/mL; 82111) and IL-4 (5 ng/mL) [80] and were treated with 0.1 µM, 1 µM and 10 µM DHA. If not otherwise mentioned, CD40 ligation was mediated by clone 82111 at 1 µg/mL. Analysis by means of flow cytometry was performed on day 6.

2.2.4.4 WST-1 assay

For studies of cell mitochondrial activity WST-1 assay was performed. Briefly, 10^5 PBMC were cultured for 4 days and stimulated with anti-CD40/IL-4. 10 µL WST-1 reagent was added during the last 3 hours of culture to each well. Cellular enzymatic activity was determined by photometrical measurement at 450 nm. Cells treated with 0.1 µM, 1 µM and 10 µM DHA were compared with DHA untreated samples. Phorbol-12-myristate-13-acetate (PMA, 10 ng/mL) was used as positive control. All experiments were performed in triplets.

2.2.5 Immunological methods

2.2.5.1 Enzyme-Linked Immuno Sorbent Assay (ELISA)

ELISA was used to quantify immunoglobulins. Thereby, the unknown amount of antigen is bound to a surface by a primary antibody. The secondary antibody is incubated over this surface so that it can bind specifically to the antigen. This detection antibody is linked to an enzyme, which converts a chromogenic substrate to some detectable signal. In case of antigen specific immunoglobulin determination primary or secondary antibody is replaced by the specific antigen. The amount of protein in the sample was calculated by means of standard curve. If not otherwise mentioned all steps were performed at room temperature.

2.2.5.1.1 Human immunoglobulin ELISA

For immunoglobulin assays cells (10^6/mL) were cultured for 10 days. Cells were stimulated with anti-CD40/IL-4 [80] and were treated with 0.1 µM, 1 µM and 10 µM DHA. After 10 days immunoglobulins were measured in cell free supernatants by ELISA. For IgE detection clones HP6061 and HP6029 were applied. The matched antibody pairs for IgA, IgG and IgM ELISA (anti-IgA, anti-IgG and anti-IgM) were purchased from Dianova. Immunoplates (maxi

Sorb) were coated overnight at 4°C with the primary antibodies for IgE clone HP6061 (2.5 µg/mL) or for the other immunoglobulins (0.4 µg/mL) diluted in sodium carbonat buffer. Unspecific binding was prevented by adding 2 % bovine serum albumine (BSA) / Tris-buffered saline (TBS) for 1 hour. Supernatants and standards were then incubated for 2 hours. After washing, the second alkaline phosphatase conjugated anti-immunoglobulin antibody (0.15 µg/mL diluted in 0.2 % BSA/TBS) was added for 1.5 hours. Since the secondary mouse anti-human IgE antibody was biotinylated (HP6029, 0.5 µg/mL diluted in 0.2 % BSA/TBS) another hour of incubation with alkaline phosphatase conjugated streptavidin (1.5 µg/mL) was performed. Following the final reaction with phosphatase substrate, plates were read in a microplate ELISA reader at 405 nm and the amount of immunoglobulin was calculated according to the standard curve. Diluted human serum of an atopic individual served as standard curve. The sensitivity of the ELISA was 100 pg/mL.

2.2.5.1.2 Murine immunoglobulin ELISA

Plates were coated overnight with anti-mouse IgE (EM95.3, 5 µg/mL) diluted in sodium carbonate buffer. After blocking with 3 % milk powder/PBS, sera (diluted in 1 % milk powder/PBS) were incubated overnight and were detected with biotin conjugated anti-mouse IgE (84 1-C, 1 µg/mL diluted in 1 % milk powder/PBS). The reaction was developed with streptavidin peroxidase and tetra methyl benzidine and was stopped with 2 N sulphuric acid. The plates were measured at 450/490 nm and total IgE was calculated according to the standard curve. The monoclonal antibodies EM95.3 and 84 1-C were kindly provided by Dr. Elke Luger, DRFZ, Berlin, Germany / Dr. Lamer, MPI, Freiburg, Gemany.

For measurement of OVA specific immunoglobulins plates were coated overnight either with rat anti-mouse IgE (R35-72, 2 µg/mL) or 5 µg/mL OVA for IgG_1 and IgG_{2a} in sodium carbonate buffer. After blocking with 3 % milk powder/PBS, serial dilution of sera in 1 % milk powder/PBS were incubated overnight, detected with biotin conjugated OVA (1.25 mg/mL, coupling rate 1:50) for IgE, with rat anti-mouse IgG_1 (A85-1, 0.5 µg/mL) or rat anti-mouse IgG_{2a} (R19-15, 0.5 µg/mL) in 1 % milk powder/PBS and developed as mentioned above. Plates were read in a microplate ELISA reader at 450/490 nm. OVA specific immunoglobulin concentrations were calculated according to the standard serum pool.

The validity of ELISAs was verified for total IgE by using serial sera dilutions and a standard of known concentration and for OVA specific immunoglobulins by using serial sera dilutions as well as by the determination of the semimaximal saturation.

2.2.5.2 Enzyme Linked Immuno Spot Technique (ELISpot)

The number of antibody secreting cells was determined by ELISpot technique. Therefore, B cells were stimulated with anti-CD40/IL-4 in the presence of 0.1 µM, 1 µM and 10 µM DHA for 7 days. 10^6 B cells were cultured for IgA and IgG, whereas $3x10^6$ cells were used for IgE ELISpot. MultiScreen-High Protein Binding Immibilion-P Membrane plates were coated overnight with mouse anti-human IgE (HP6061, 2.5 µg/mL) and with goat anti-human IgA or IgG (0.4 µg/mL, Dianova) in sodium carbonate buffer. Unspecific binding was blocked by adding 2 % BSA/PBS for 1 hour. After several washings, serial dilutions of B cells from 7 day cultures were incubated at 37°C for adequate period of time. After washing, detection was performed with biotin conjugated antibodies: mouse anti-human IgE (HP6029, 1.5 µg/mL), mouse anti-human IgA (G20-359, 5 µg/mL) or mouse anti-human IgG (G18-145, 0.1 µg/mL) in 0.2 % BSA/PBS. The reaction was developed with streptavidin-horseradish peroxidase (0.75 µg/mL) and peroxidase substrate 33-Amino-9-ethyl-carbazole/ n,n-Dimethyl formamide. Antibody secreting cells appeared as red spots and were counted with CTL ImmunoSpot® S4.

2.2.5.3 Flow cytometry

Fluorescence activated cell sorting (FACS) enables the analysis of single cells due to their characteristical light scattering and their emitted fluorescence. By the mean of forward light scatter (FSC) / side light scatter (SSC) characteristics, i.e. size and granularity, it is possible to distinguish between different cell populations. Analysis of specific intracellular and extracellular antigens can be performed by staining with fluorescence dye (e.g. FITC [Fluoresceine isothiocyanat], PE [Phycoerythrine], APC [Allophycocyanine]) coupled antibodies. In flow cytometer these dyes are excited by appropriate laser and subsequently emit light of different wave lengths. This emission is measured by defined detectors and is depicted in a graph as intensity per cell (Figure 11).

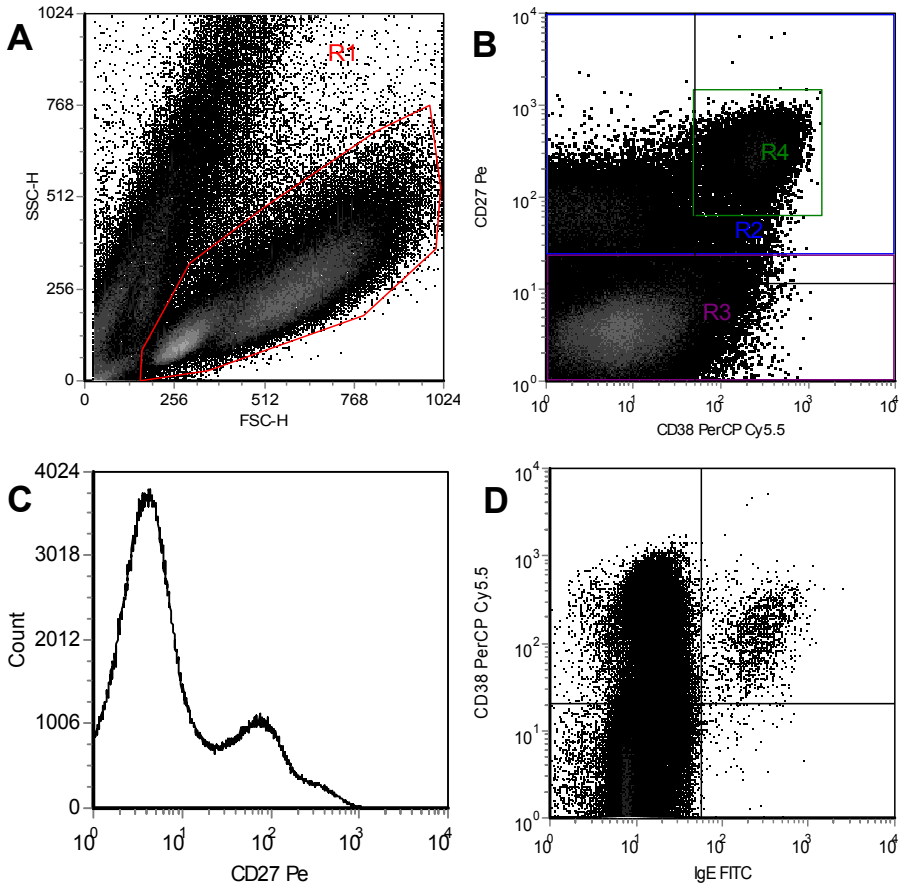

Figure 11: Exemplary flow cytometry images. A) FSC / SSC characteristics of B cells after 7 days of stimulation with anti-CD40/IL-4/IL-21, **B)** dot blot of anti-CD27 plus anti-CD38 stained, **C)** histoblot of anti-CD27 stained and **D)** dot blot of anti-CD38 plus anti-IgE stained B cells.

2.2.5.3.1 Flow cytometric analysis of PBMC activation

Within the clinical trial the effect of DHA supplementation on activation status of PBMC isolated cells was investigated. Therefore, isolated PBMC were stained with a combination of fluorescence dye labelled monoclonal antibodies. Antibodies used were anti-CD19 (4G7), anti-CD14 (MϕP9), anti-CD23 (EBVCS-5) and anti-HLA-DR (Human Leukocyte Antigene-DR, L243). Staining was performed for 15 min at 4°C in the dark.

2.2.5.3.2 Flow cytometric analysis of cytokines in PBMC

Further on, the interference of DHA supplementation with systemic cytokine response was investigated in PBMC of treated patients. Therefore, $2x10^6$ PBMC were cultured in the presence of Staphylococcus Enterotoxine B (SEB; 1 µg/mL) and anti-CD28 (L293, L25; 0.2 µg/mL) or without any stimulation for 24 hours. To accumulate synthesised proteins in-

tracellularly, 1 µg/mL Brefeldin A was added 4 hours before expiration of the incubation. After harvesting, cells were stained with fluorescence dye labelled monoclonal anti-human CD4 (SK3) and anti-CD69 (L78) for 15 min at 4°C in the dark. After the following washing step, PBMC were fixed with 2 % paraformaldehyde (PFA) for 15 min at room temperature. Staining of intracellular cytokines was performed with anti-human IFNγ (4S·B3) and anti-IL-4 (8D4-8) in 0.5 % saponin / FACS buffer for 35 min at 4°C. Cells were immediately analysed at the flow cytometer. At least 100000 gated cells were collected for each sample.

2.2.5.3.3 Flow cytometric analysis of STAT6 phosphorylation

Within the scope of in vitro investigations, the impact of DHA on the IL-4 signalling pathway STAT6 phosphorylation was investigated by flow cytometry. After starving in the presence of 0.1 µM, 1 µM and 10 µM DHA overnight, isolated B cells were stimulated with 10 ng/mL IL-4 for 1 hour and harvested into BD Phosflow Fix Buffer I according to manufacturer's instructions. Unstimulated cells were harvested in a similar fashion. After fixing the cells for 10 min at 37°C, B cells were permeabilised with Phosflow Perm Buffer III for 30 min on ice. After washing, cells were stained with anti-pSTAT6 (pY641; clone 18), anti-STAT6 (clone 23), anti-CD27 (LG.7F9) and anti-CD38 (HIT2) for 1 hour at room temperature. Cells were immediately analysed at the flow cytometer. At least 30000 gated B cells were collected for each sample and geometric mean fluorescence was analysed.

2.2.5.3.4 Flow cytometric analysis of IκBα degradation

IkBα degradation was measured by flow cytometry to disclose the mechanisms of DHA mediated inhibition of p50 translocation. $CD19^+$ B cells were preincubated with 0.1 µM, 1 µM and 10 µM DHA for 2 hours followed by a stimulation with 1 µg/mL anti-CD40 (G28.5) for 1 hour. Subsequently, stimulated and unstimulated cells were harvested directly into 4 % PFA. After fixing the cells for 10 min at 37°C, B cells were incubated for 15 min with anti-CD20 (2H7), anti-CD27 (LG.7F9) and anti-CD38 (HIT2) on ice. After an additional washing step, cells were stained with anti-IκBα (L35A5) in 1 % saponin / FACS buffer for 30 min at 4°C. Cells were immediately analysed at the flow cytometer. At least 30000 gated B cells were collected for each sample and geometric mean fluorescence was analysed.

2.2.5.4 Immunohistochemistry

Using biopsy punches, 5 mm diameter sections were obtained from patch areas of murine bellies, embedded in freezing medium and carefully frozen into liquid nitrogen. The tissue was cut into 5 µm specimens by a microtome at about -30°C and directly transferred on mi-

croscope slide. After drying on a hot plate, samples were stored at -80°C until further preparation.

2.2.5.4.1 Staining of $CD4^+$ and $CD8^+$ T cells

Initially, unspecific binding was blocked by using 5 % goat serum in TBS for 20 min and application of the Avidin / Biotin Blocking Kit according to manufacturer's instructions. After 1 hour of incubation with rat anti-mouse CD4 (L3T4, 15 µg/mL, Figure 12A) or CD8 (53-6.7, 15 µg/mL, Figure 12B), sections were exposed to biotinylated goat anti-rat IgG over 30 min. Staining was developed using Dako REAL™ Detection System Alkaline Phosphatase / RED. Thereby, alkaline phosphatase is covalently bound to biotin of secondary antibody by a streptavidine bridge. The subsequently converted substrate designates the positive cells. Haematoxylin was used for nuclei counterstaining. This staining is based on the building of chelate rings of haematin plus salts with phosphate groups of nucleic acid in nucleus. The whole specimen was afterwards mounted on glass slides with preheated Kaiser's glycerin jelly. All steps were performed at room temperature.

2.2.5.4.2 Mast cell staining

To visualise MC granula filled with heparin and histamine (metachromatic), samples were stained with 0.1 % toluidine blue in 0.5 N HCl for 1 hour followed by short washing step with tap water (Figure 12C).

2.2.5.4.3 Measurement of epidermal thickening

Thickness of epidermis was determined by Axiovision measuring tools on the Axioplan light microscope at x100 magnification. The measurement included the stratum granulosum, stratum spinosum and stratum basale. Since stratum corneum was partly damaged due to the procedure of fixing and slicing of the skin, its thickness could not be determined in all cases and was therefore excluded. For each mouse, eight measurements were obtained (Figure 12D).

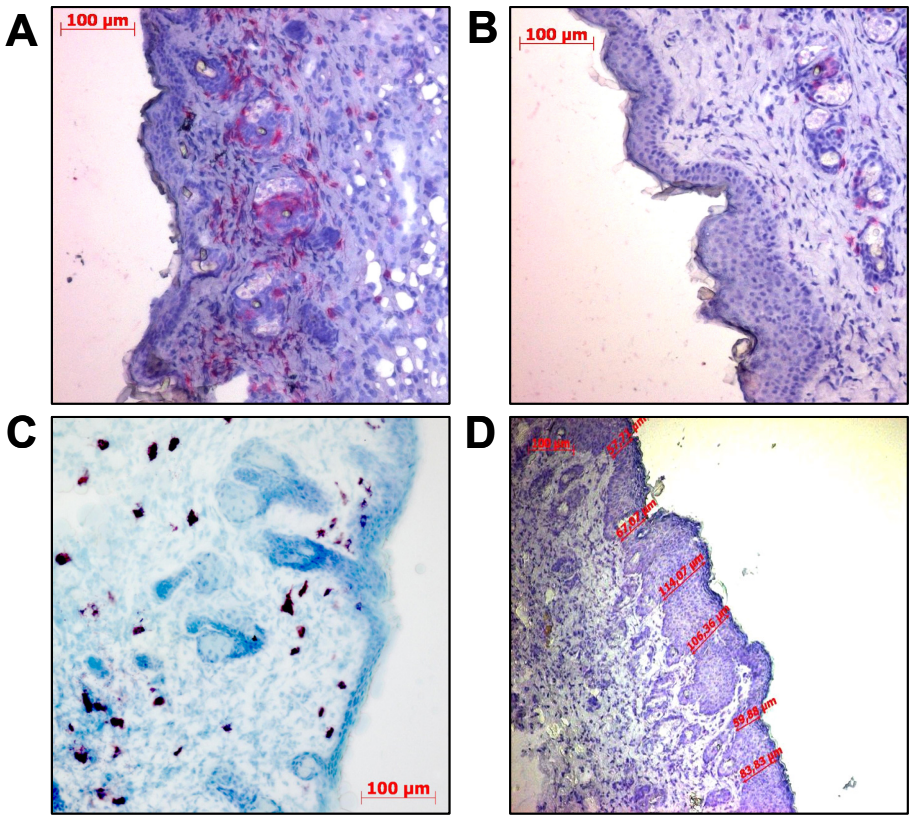

Figure 12: Illustration of histological analysis of protein induced dermatitis in BALB/c mice. Cell infiltrates of **A)** $CD4^+$, **B)** $CD8^+$ and **C)** MC as well as **D)** skin thickness were determined in 5 µm section of frozen skin samples. Positive immunohistochemical stained cells were counted in 100 µm x 200 µm areas at x100 magnification using the measuring tools of Axiovision software. Skin thickness (µm) was determined in haematoxylin stained skin site by digital measurement tools.

2.2.5.4.4 Quantification of cellular infiltrates

To record dermal infiltration of $CD4^+$, $CD8^+$ cells and MC, positive stained cells were counted in 100 µm x 200 µm areas at x100 magnification using measuring tools of Axiovision software. Only cells in the dermis were counted by positioning the square lengthwise on the border of epidermis and dermis. The mean of eight sections per mouse was calculated.

2.2.6 Molecular biological methods

For gene expression analysis real-time reverse-transcriptase (RT) polymerase chain reaction (PCR) was performed. Therefore RNA was isolated, transcribed into complementary DNA (cDNA) and finally analysed by quantitative PCR.

2.2.6.1 RNA isolation

For analysis of εGLT and AID mRNA expression 10^6 B cells were stimulated with anti-CD40 and / or IL-4 in the presence of 0.1 µM, 1 µM and 10 µM DHA for 4 days.

RNA isolation of single cell suspensions was performed using RNA isolation kit Nucleospin®RNA II according to manufacturer's guidelines. This technique bases on a two column system. The first shredder column removes firm components from nucleic acids and the second column binds nucleic acids. Salts, metabolites and macromolecular cellular components are removed by several washing steps with different buffers.

Briefly, 10^6 cells were directly lysed by adding 350 µL RA1 buffer with 1 % beta mercapto ethanol. This lysis buffer immediately inactivates RNases and generates optimal conditions for adsorption of RNA to the silica membrane. Contaminating DNA was removed by a 15 min rDNase digestion. After numerous washing steps, RNA is eluted in RNase free water and its concentration is photometric measured at 260 nm in ND-1000 Spectrophotometer. To achieve higher RNA concentrations, the visible dye labeled carrier Pellet Paint™ Co-Precipitant was used in combination with alcohol precipitation method. Pellets were resuspended in RNase free water for cDNA synthesis.

2.2.6.2 cDNA synthesis

Synthesis of cDNA was performed with Taq Man Reverse Transcription Reagent according to manufacturer's instructions. This kit contains MultiScribe® Reverse Transcriptase, a recombinant Moloney Murine Leukemia Virus Reverse Transcriptase, random hexamers and oligo (dT). Finally, cDNA was stored at -20°C.

2.2.6.3 Quantitative PCR

For gene expression analysis real-time RT-PCR was performed. After thermal denaturation of double stranded DNA into single stranded DNA (95°C), oligonucleotides (primer) can complementarily hybridise to a specific sequence on each strand of the target DNA at a primer dependent "annealing" temperature (60 to 65°C). Afterwards, a thermo stable DNA polymerase prolongs the oligonucleotides (72°C). The newly generated DNA sequence matches the sequence following the primer. After synthesising new DNA, the same primers will be released and used again. This gives DNA a logarithmic amplification.

By the mean of double stranded DNA binding dye chemistry the amplicon production was quantified by using a non-sequence specific fluorescent intercalating agent. SYBR Green is a fluorogenic minor groove binding dye that does not bind to single stranded DNA. This dye

emits a strong fluorescent signal upon binding to double stranded DNA whereas it exhibits only little fluorescence in solution. Thus, as PCR products accumulate, fluorescence increases. Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle, i.e. in real time. Melting curve analysis was used to identify the specific amplicon and to exclude non-specific amplifications. All samples were measured at least in duplicates. Water was used as negative control.

The used oligonucleotides (primer) were designed by means of the internet databases UCSC and NCBI and created by online software Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The required parameters for well designed primers and samples have been documented and are built into the program. These parameters include a melting temperature for the sample that is 10°C higher than the primers, primer melting temperature about 60°C, amplicon size between 80 and 150 bases, absence of 5' guanosines and primer length between 20 and 25 nucleotides. The best design for primers for the quantification of mRNA expression requires intron spanning positioning. To achieve most optimal and specific results, PCR conditions were optimised regarding primer concentration, annealing temperature and time, magnesium chloride concentration as well as elongation time. The depicted primers are applied at a hybridisation temperature of 65°C and at concentration of 500 nM (Table 3).

Gene	5' - 3' Sequence	amplicon [bp]	$MgCL_2$ [mM]	Efficiency E
HPRT for	TggCTTATATCCAACACTTCgTg	187	4	2.00
HPRT rev	ATCAgACTgAAgAgCTATTgTAATgACCA			
εGLT for	gACgggCCACACCATCCACAggCAC-CAAATggACgAC	409	5	1.91
εGLT rev	CAggACgACTgTAAgATCTTCACg			
AID for	AgAggCgTgACAgTgCTACA	93	4	1.88
AID rev	ATgTAgCggAggAAgAgCAA			

Table 3: Used oligonucleotides (primer) for quantitative real-time PCR.

For quantitative real-time PCR LightCycler-Fast Start *Master* SYBR Green I was used according to manufacturers' guidelines. The reaction volume was reduced to 5 µL per sample.

While reaction mass is denaturating for 10 min at 95°C, hot start polymerase is activated in the LightCycler unit. The measurement of the fluorescent signal is performed directly after elongation by polymerase. Thus, amplification progress can be detected simultaneously with

intercalation of SYBR Green. Amplification of a given cDNA over time follows a curve, with an initial flat phase, followed by an exponential phase. As the experiment reagents are used up, DNA synthesis slows and exponential curve flattens into a plateau. For the RT-PCR principle, the more cDNA is in a sample, the earlier it will be detected during repeated cycles of amplification.

Target gene levels were normalised to a housekeeping gene as an endogenous control, to correct potential variation in RNA loading, cDNA synthesis or efficiency of the amplification. Expression levels of reference genes should remain roughly constant between cells of different tissues and under different experimental conditions. Here hypoxanthine guanine phosphoribosyltransferase (HPRT) was used to evaluate expression of target genes.

The comparative C_P method for analysis involves comparing the threshold cycle or crossing point (C_P) values of samples of interest with a control. The relative expression ratio (R) of a target gene is based on the real-time efficiency (E) of the primer pair, the C_P deviation of an unknown sample versus a control and is expressed in comparison to a reference gene (ref) (Formula 1). Either DHA untreated sample in in vitro experiments or control diet fed mice were set as control. Efficiency corrected relative quantification was performed by 2-$\Delta\Delta C_P$ method [81].

$$R = \frac{(E_{\text{target}})^{\Delta C_P \text{target(control-sample)}}}{(E_{\text{ref}})^{\Delta C_P \text{ref(control-sample)}}}$$

Formula 1: ΔΔCP method for mRNA quantification.

2.2.6.4 Western blot

The western blot technique enables the detection of a specific protein in a given sample homogenate or extract. Primarily, gel electrophoresis is used to separate native or denatured proteins by the length of the polypeptides. The proteins are transferred afterwards to a membrane (typically nitrocellulose or polyvinylidene difluoride [PVDF]), where they are detected using antibodies specific for target protein. Here, western blot is used to investigate the impact of DHA on the NFκB signalling by the means of p50 translocation into nucleus.

After 2 hours preincubation with 10 µM DHA, isolated B cells were stimulated with 1 µg/mL anti-CD40 (G28.5) for 1 hour. Protein extracts of $5x10^6$ B cells were prepared by NE-PER extraction reagent containing a protease inhibitor cocktail and were subsequently quantified by standard coomassie plus protein assay according to manufacturer's instructions. Nuclear

extracts (3 to 5 µg) were separated in a 12 % tris glycine sodium dodecyl sulfate (SDS) polyacrylamide gel. Proteins were transferred to PVDF membrane and blocked for 1 hour with 5 % milk powder/PBS. Overnight membrane was incubated with 0.2 µg/mL anti-human p50 (H119) in 1 % milk powder/PBS-Tween (PBST) at 4°C. After washing the membrane with PBST, horse radish peroxidase (HRP) conjugated goat anti-rabbit IgG (0.1 µg/mL in 1 % milk powder/PBST) was added for 1 hour at room temperature. The antigen detection was performed with the chemiluminescent detection system ECL Plus.

2.3 Statistical analysis

Wilcoxon test for non-parametrical, paired data using SPSS (***S**tatistical **P**ackage for the **S**ocial **S**ciences)* software package for Windows, Release 12.0 and 14.0 was carried out to analyse the statistical significance of within-group changes. Non-parametrical, unpaired data was analysed by Mann-Whitney U-test to evaluate the significance of between group changes. A p-value < 0.05 was considered to be statistically significant.

3 Results

3.1 DHA inhibits IgE production in human B cells

3.1.1 Modulation of immunoglobulin synthesis

3.1.1.1 DHA inhibits anti-CD40/IL-4 mediated IgE production in B cells

To investigate the impact of DHA on IgE production, PBMC and B cells were analysed regarding IgE production and number of IgE secreting cells using ELISA and ELISpot, respectively. For better illustration DHA untreated stimulated sample was set as 100 %.

IgE production from PBMC of non-allergic donors was negligible (< 20 pg/mL). After anti-CD40/IL-4 stimulation, in supernatants IgE increased up to 7378 ± 4475 pg/mL ($p = 0.028$). DHA treatment led to a dose-dependent reduction of IgE production. 10 µM DHA was most efficient to inhibit reaching 714 ± 382 pg/mL ($p = 0.028$) (Figure 13A).

PBMC of allergic donors showed a basal IgE production up to 3400 pg/mL which was increased by anti-CD40/IL-4 stimulation to 16206 ± 3758 pg/mL ($p = 0.028$). Regarding their response to DHA treatment two subgroups among atopic patients were identified. PBMC from donors with a high basal IgE production (IgE > 1000 pg/mL) did not respond to DHA (data not shown). In contrast, in PBMC cultures of donors with basal IgE production lower than 1000 pg/mL DHA caused a dose-dependent reduction of IgE production as noticed in cultures of healthy individuals. Thereby, 10 µM DHA caused the strongest reduction to 1182 ± 838 pg/mL ($p = 0.028$) (Figure 13A).

Next it was investigated whether the inhibition of IgE was due to direct action of DHA on B cells. After anti-CD40/IL-4 stimulation, IgE production of B cells increased up to 25-fold (2270 ± 1610 pg/mL, $p < 0.001$) and was dose-dependently inhibited by DHA treatment. The highest DHA concentration caused the strongest inhibition as measured with 485 ± 719 pg/mL IgE in supernatants ($p < 0.001$, Figure 13B).

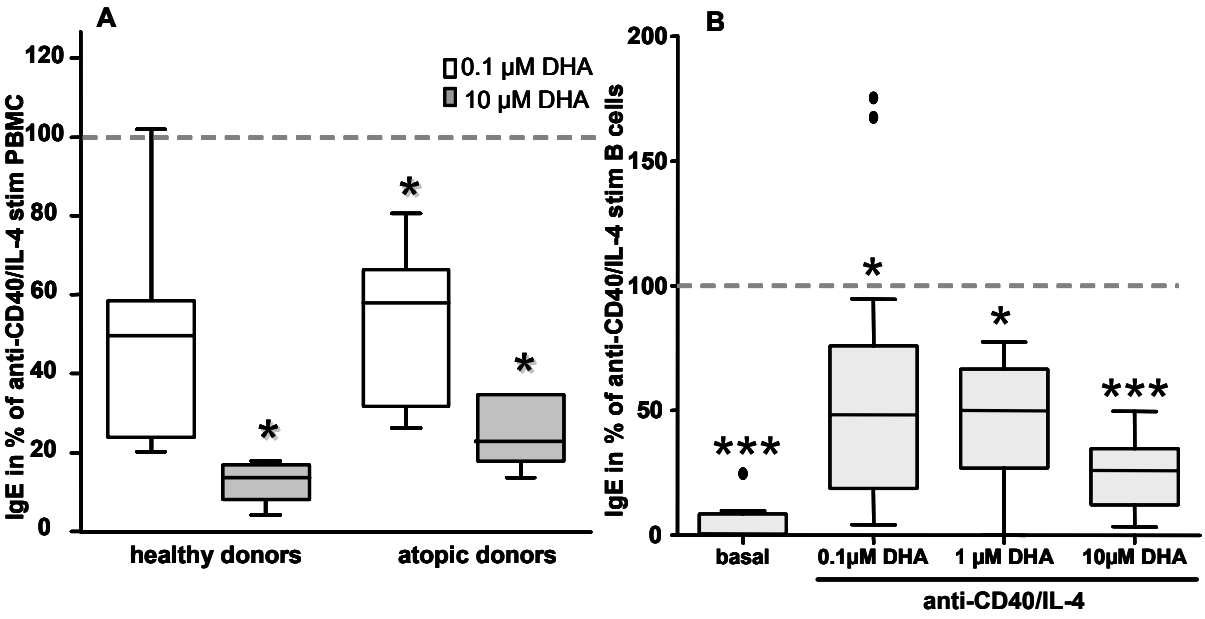

Figure 13: DHA inhibits IgE production dose-dependently in anti-CD40/IL-4 stimulated A) PBMC and B) B cells. Isolated PBMC or B cells were stimulated with anti-CD40/IL-4 for 10 days. IgE was determined in supernatants by ELISA. IgE production in % of anti-CD40/IL-4 stimulated, DHA untreated cells (100 %). Data are shown as box plot (25. and 75. percentile, median); n ≥ 6; * statistically significant difference compared to DHA untreated sample (* $p < 0.05$; *** $p < 0.001$).

To delineate whether DHA interferes with the early switch machinery or the late immunoglobulin secretion process, DHA was added to B cell culture at different time points (days 0, 2, 4, 6 and 8). The results show that DHA exerted the strongest IgE inhibition when added at the beginning of the culture. This inhibition was continuously decreasing if added at later time points (Figure 14).

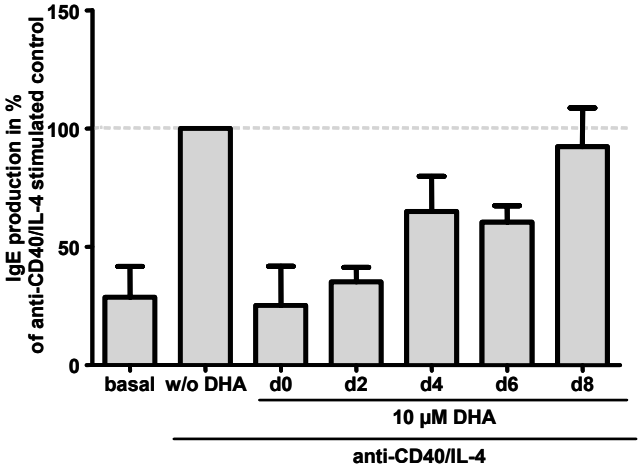

Figure 14: DHA inhibits early switching. Experimental setting is described in Figure 13. 10 µM DHA was added to B cell culture at different time points (days 0, 2, 4, 6 and 8). IgE production in % after treatment with 10 µM DHA relative to anti-CD40/IL-4 stimulated, DHA untreated sample (100 %). Data are shown as mean + SD; n = 3.

Furthermore, IgA and IgG production was measured in supernatants to examine whether the inhibition of IgE was isotype specific. Indeed, DHA treatment did not alter IgA and IgG production of cultured B cells (Figure 15).

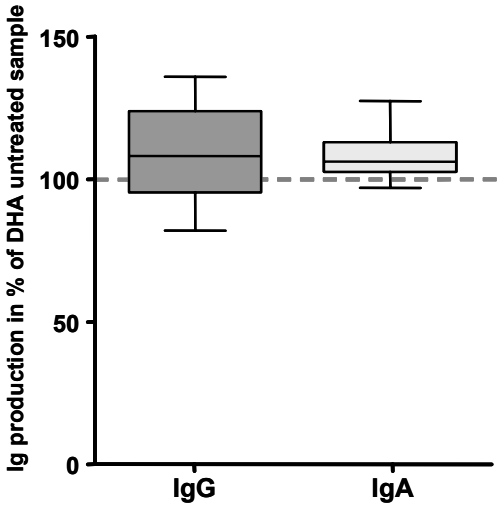

Figure 15: DHA does not affect IgG and IgA production in B cells. Experimental setting is described in Figure 13. Immunoglobulin (Ig) production in % after treatment with 10 µM DHA relative to anti-CD40/IL-4 stimulated, DHA untreated sample (100 %). Data are shown as box plot (25. and 75. percentile, median); n ≥ 6.

3.1.1.2 DHA reduces the number of IgE secreting cells

To prove that the reduced amount of IgE in supernatants was due to a reduced number of antibody secreting cells, ELISpot analysis was performed after 7 days culture.

As depicted in Figure 16A, DHA treatment of B cell cultures caused a concentration-dependent effect on IgE secreting cells. 10 µM DHA reduced significantly the number of IgE secreting cells (51 ± 34 %, p = 0.043). Interestingly, 0.1 µM DHA did not decrease, but rather increased IgE secreting cell numbers (139 ± 30 %, p = 0.043). 100 % correspond to 75 ± 79 IgE secreting cells / 10^6 B cells.

In contrast, DHA did not reduce the number of IgA secreting cells (Figure 16B). But, 10µM DHA slightly reduced the number of IgG secreting cells to 91 ± 25 % of DHA untreated sample (p = 0.043, Figure 16C). 343 ± 284 IgG secreting cells / 10^6 B cells and 237 ± 180 IgA secreting cells / 10^6 B cells represent 100 %.

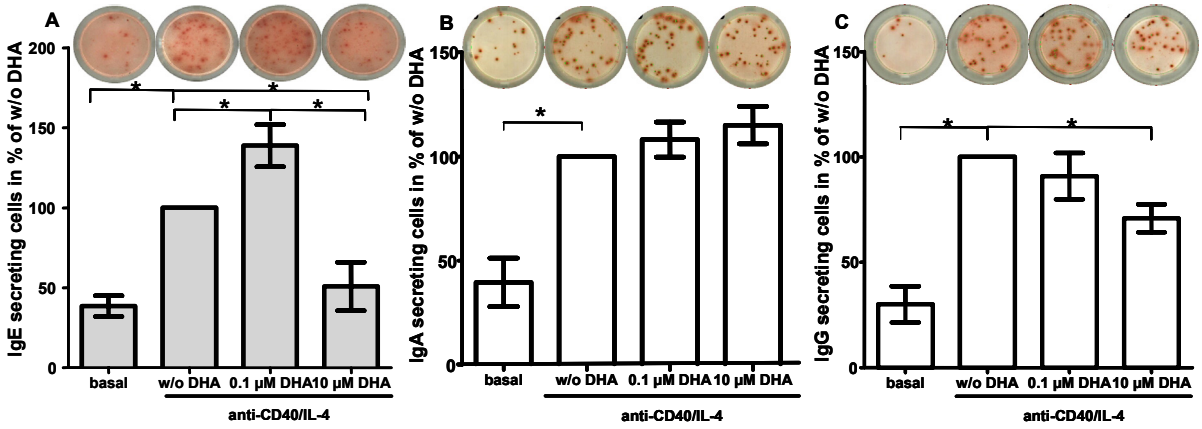

Figure 16: DHA reduces the generation of A) IgE secreting cells in anti-CD40/IL-4 stimulated B cells, whereas B) IgA was not affected and C) IgG was slightly reduced only at the highest DHA concentration. B cells were stimulated with anti-CD40/IL-4 for 7 days. Number of antibody secreting cells was determined by ELISpot. Data are shown in % of anti-CD40/IL-4 stimulated, DHA untreated control (100 %), as mean ± SD; n ≥ 5; * p < 0.05 statistically significant difference compared to anti-CD40/IL-4 stimulated, DHA untreated sample.

Next, antiproliferative or toxic effects mediated by DHA were ruled out. As shown in Figure 17, DHA exerted no inhibition of B cell proliferation as measured by CFSE labelling. Furthermore, toxicity by DHA was excluded by tryptan blue and propidium iodide staining.

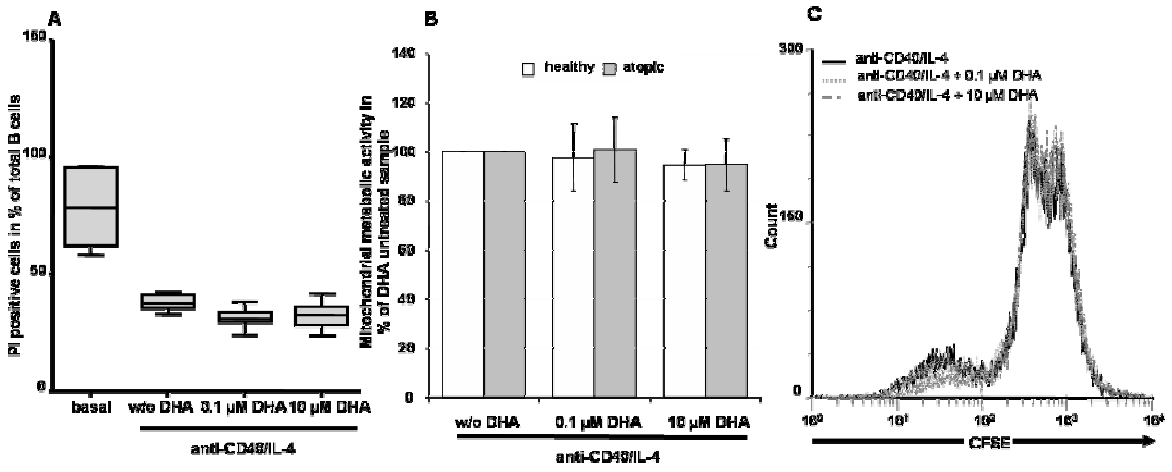

Figure 17: DHA is not toxic or antiproliferative in B cells. A) Propidium iodide (PI) positive B cells after 10 days of stimulation with anti-CD40/IL-4 with or without DHA. Data are shown as box plot (25. and 75. percentile, median); n = 5. **B)** Mitochondrial metabolic activity after 3 days of stimulation with anti-CD40/IL-4 with or without DHA as measured by WST-1. Data are shown as mean ± SD; n = 8. **C)** CFSE division after 4 days of stimulation with anti-CD40/IL-4 in the presence or absence of DHA. Histogram is a representative of three independent experiments; n = 3.

3.1.1.3 DHA reduces activation-induced (cytidine) deaminase (AID) mRNA expression

Since AID is essential for isotype switching [17], the impact of DHA on its expression was determined next.

As shown in Figure 18A, AID mRNA expression was induced by anti-CD40/IL-4 stimulation up to fivefold ($p = 0.043$) and decreased dose-dependently in DHA treated B cells. The strongest inhibition was detected when the highest DHA concentration was used (44 ± 32 %, $p = 0.018$). However, the overall inhibition was less prominent as DHA mediated εGLT inhibition.

3.1.1.4 DHA inhibits IgE switch recombination

Immunoglobulin heavy chain CSR correlates with the induction of specific germline C_H transcripts. The expression of εGLT precedes IgE production and indicates switching process towards IgE [82,83].

In B cells εGLT expression was induced fivefold by anti-CD40/IL-4 stimulation ($p = 0.043$). The presence of DHA led to a dose-dependent reduction of εGLT expression. The maximal inhibition was observed with 10 µM DHA reaching 87 ± 13 % ($p = 0.028$) (Figure 18B).

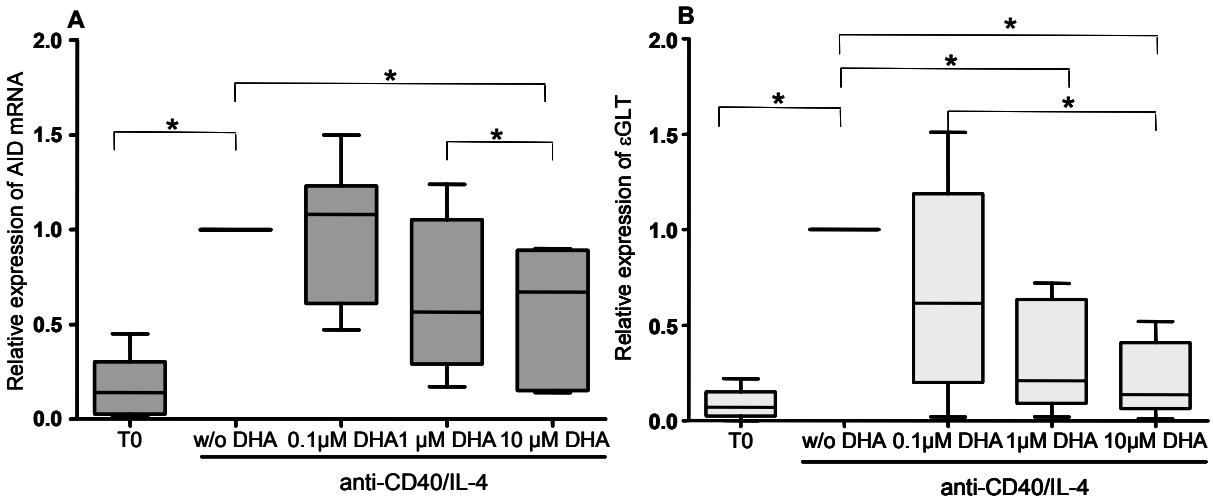

Figure 18: DHA inhibits A) AID and B) εGLT expression in anti-CD40/IL-4 stimulated B cells in a dose-dependent manner. B cells were stimulated with anti-CD40/IL-4 and treated with DHA. After 4 days of incubation cells were harvested and AID and εGLT mRNA expression was determined using quantitative RT-PCR. Fold expression of anti-CD40/IL-4 stimulated, DHA untreated control. Data are shown as box plot (25. and 75. percentile, median); n = 8; * $p < 0.05$ statistically significant difference compared to anti-CD40/IL-4 stimulated DHA untreated sample.

To delineate whether the IL-4 and / or the CD40 pathway was disrupted by DHA, B cells were stimulated with anti-CD40 or IL-4 alone. Thereby 10 µM DHA inhibited εGLT expression induced by IL-4 (51 ± 31 %, $p = 0.018$) as well as by CD40 ligation (52 ± 1 %, $p = 0.001$) dose-dependently (data not shown).

3.1.2 DHA modulates IL-4 and anti-CD40 signalling

3.1.2.1 DHA reduces STAT6 phosphorylation

Since εGLT and AID were inhibited by DHA and their promoter regions contain IL-4 dependent STAT6 and CD40 dependent p50 binding sites [5], DHA mediated IgE inhibition might be attributed to the modulation of CD40 and / or IL-4 signalling.

IL-4 signalling regulates transcription of IL-4 responsible genes through STAT6 phosphorylation and dimerisation [9]. Phosphorylation of STAT6 (pSTAT6) in the presence of DHA was measured by flow cytometry.

The basal phosphorylation level of STAT6 was low (mean fluorescence intensity [MFI] 3.0 ± 0.6). Whereas IL-4 stimulation increased the number of pSTAT6 positive cells 50-fold

and the MFI threefold (11.0 ± 3.7, p = 0.008). As depicted in Figure 19, DHA reduced STAT6 phosphorylation dose-dependently at 1 µM (MFI 9.3 ± 4.4, p = 0.022) and 10 µM DHA (8.1 ± 3.6, p = 0.008). However, 0.1 µM DHA did not affect STAT6 phosphorylation (11.6 ± 4.2, n.s.).

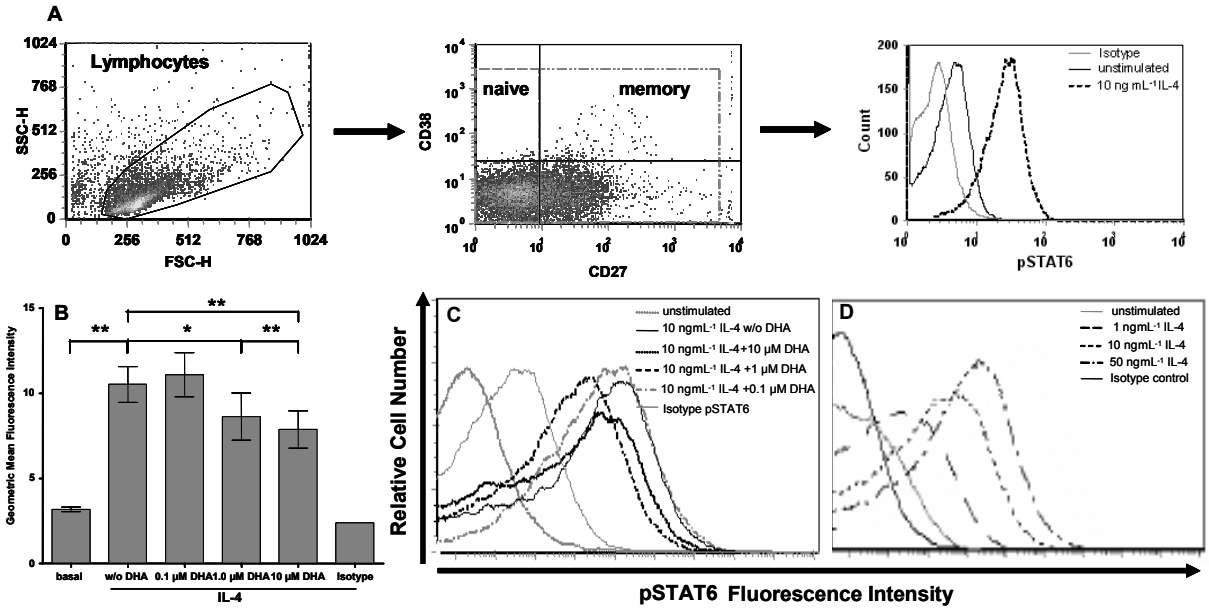

Figure 19: DHA inhibits phosphorylation of STAT6 dose-dependently in IL-4 stimulated B cells. After starving in the presence of DHA overnight, B cells were stimulated with IL-4 for 1 hour. After fixing the cells, cells were permeabilised and stained with anti-pSTAT6, anti-STAT6, anti-CD27 and anti-CD38. At least 30000 gated B cells were collected for each sample. The geometric mean fluorescence intensity was analysed. **A)** Gating strategy; **B)** Data are shown as mean ± SD; n = 8; **C)** Representative histogram for DHA treatment; **D)** Representative histogram for dose-response analysis; * p < 0.05; ** p < 0.01 statistically significant difference compared to IL-4 stimulated, DHA untreated sample.

This reduction was not restricted to B cell subsets as both naïve or memory B cells exhibited reduced STAT6 phosphorylation (data not shown). Concomitantly, a decreased ratio between STAT6 and pSTAT6 was measured (p = 0.018, Figure 20). Importantly, DHA treatment did not inhibit STAT6 protein expression of B cells.

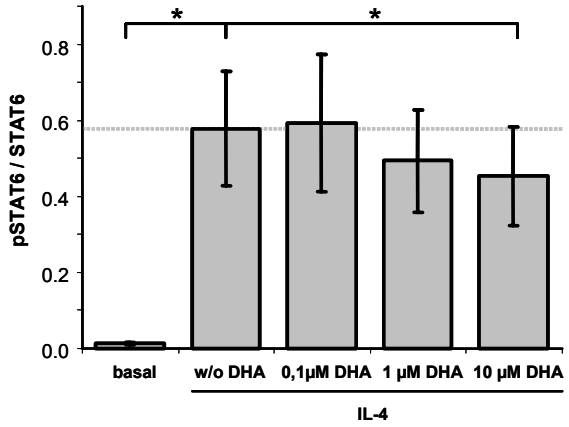

Figure 20: DHA decreases pSTAT6 and STAT6 ratio. Experimental setting is described in Figure 19. Data are shown as mean ± SD; n = 8; * $p < 0.05$ statistically significant difference compared to IL-4 stimulated, DHA untreated sample.

3.1.2.2 DHA decreases NFκB p50 translocation

NFκB p50 is the most relevant downstream event of CD40 involved in isotype switching [15]. To analyse whether the detected impairment of IgE production is accompanied by a decreased p50 translocation, nuclear protein expression in DHA treated B cells was examined by western blot technique.

As shown in Figure 21, p50 protein was detectable in nuclear extracts of unstimulated B cells. Stimulation with anti-CD40 caused the additional translocation of p50 into the nucleus from 129 ± 20 to 177 ± 29 densitometric arbitrary units ($p = 0.018$). However, concomitant treatment with 10 µM DHA inhibited anti-CD40 driven nuclear p50 translocation (125 ± 41 densitometric arbitrary units; $p = 0.028$).

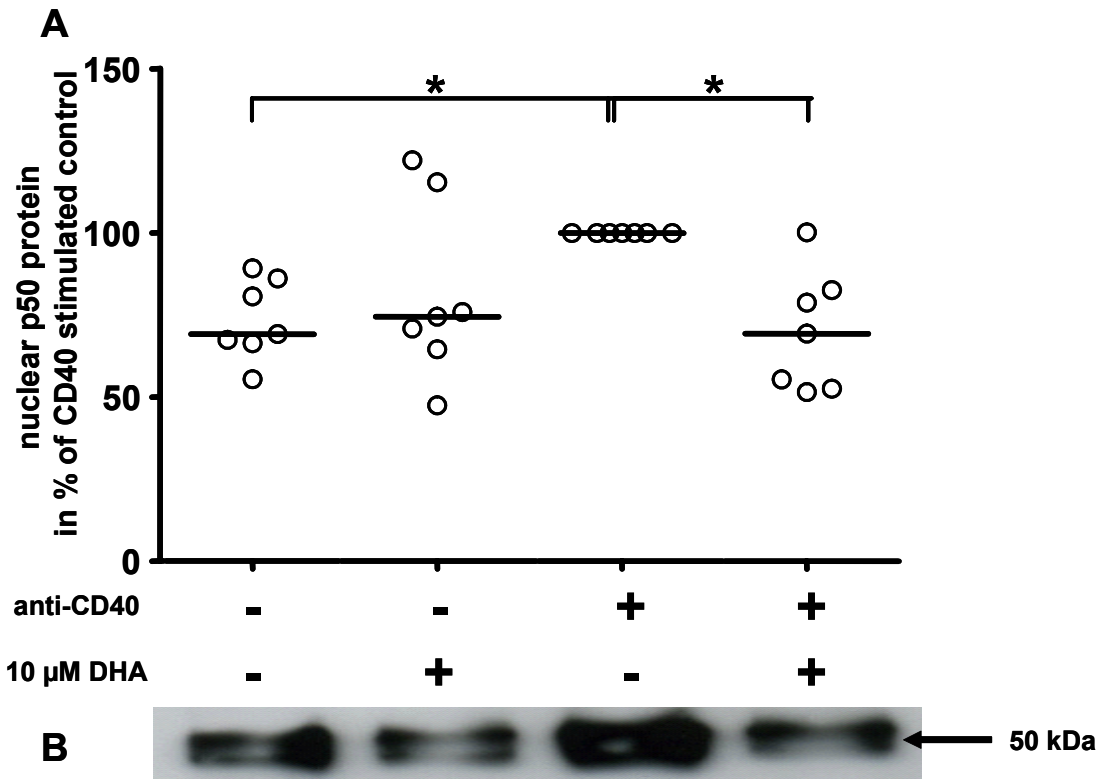

Figure 21: DHA inhibits anti-CD40 induced p50 translocation into the nucleus in B cells. Isolated B cells were preincubated with 10 µM DHA for 2 hours and subsequently stimulated with anti-CD40 for 1 hour. Nuclear extracts were analysed by western blot. p50 protein was detected at 50 kDa. **A)** Data are shown as single values, bars represent mean values. **B)** Blot is a representative of seven independent experiments (n = 7); * $p < 0.05$ statistically significant difference compared to anti-CD40 stimulated, DHA untreated sample.

Finally, it was determined whether a modulation of IκBα degradation by DHA was responsible for the inhibited p50 translocation. The intracellular IκBα content decreased from a basal geometric MFI of 172 ± 19 to a MFI of 92 ± 26 upon anti-CD40 stimulation. These data rule out such a mechanism as DHA did not alter the anti-CD40 driven degradation of IκBα measured by flow cytometry (Figure 22).

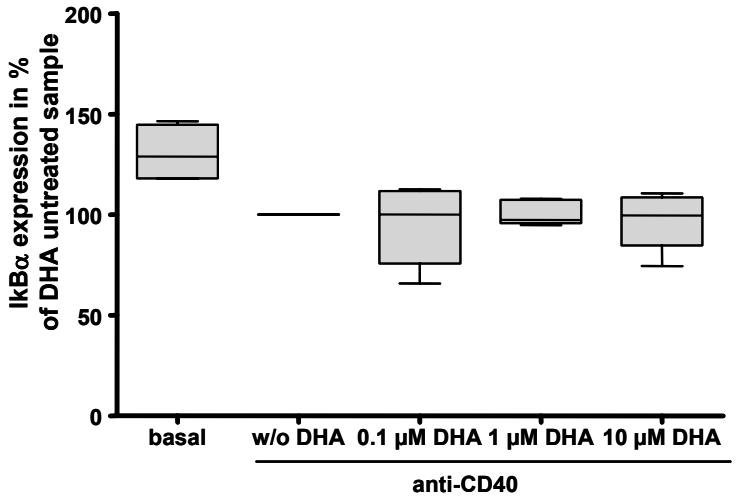

Figure 22: DHA does not affect anti-CD40 driven IκBα degradation. Experimental setting is described in Figure 21. B cells were stained with anti-CD20, anti-CD27, anti-CD38 and anti-IκBα. 30000 gated B cells were collected for each sample and the geometric mean fluorescence was analysed. Data are shown as box plot (25. and 75. percentile, median); n = 6.

3.2 DHA supplementation in atopic eczema – a randomised, double blind, controlled study

According to the in vitro data, DHA caused a significant and dose-dependent inhibition of IgE production in anti-CD40/IL-4 stimulated PBMC and exhibited immunomodulating properties. Therefore, the therapeutic efficacy of dietary DHA was investigated in a randomised, controlled and double blind clinical trial with atopic eczema patients. The tolerability of DHA was excellent. Only three participants reported slight gastrointestinal discomfort.

3.2.1 The SCORAD is significantly reduced by DHA supplementation

As shown in Figure 23, a significant reduction of the SCORAD was observed in the DHA treated group ($p = 0.009$), but not in the control group. However, in both groups an improvement of SCORAD was detected. In the DHA group the assessed median decline of SCORAD was -18 % [-96 % - 29 %], whereas in the control group the SCORAD decreased -11 % [-84 % - 35 %] from baseline to week 8.

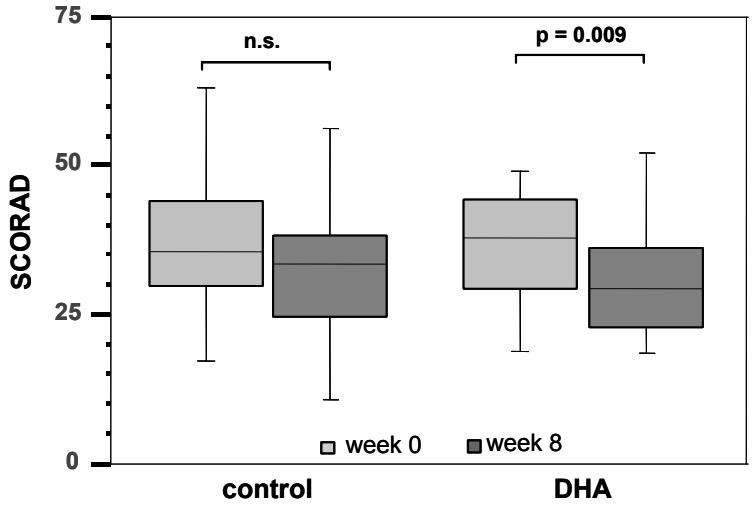

Figure 23: **SCORAD is significantly reduced by dietary DHA.** SCORAD was determined in control (n = 23) and DHA (n = 21) group during the course of supplementation by the same physician. Data are shown as box plot (25. and 75. percentile, median) [84].

The decreased SCORAD in the DHA group was mainly attributed to the significant reduction of affected areas from 12 [2 - 41] SCORAD points at baseline to 7 [2 - 25] at week 8 ($p = 0.02$; data not shown). No change of individuals' medication usage occurred during supplementation.

3.2.2 DHA inhibits IgE synthesis ex vivo

Before and after supplementation the IgE production of stimulated PBMC was measured in order to determine the impact of fatty acid supplementation on IgE synthesis. At baseline IgE production in anti-CD40/IL-4 stimulated PBMC was comparable in both groups and decreased only during DHA treatment significantly (week 0: 10.62 ± 9.2 ng/mL; week 8: 3.6 ± 3.1 ng/mL; $p = 0.013$; Figure 24). By contrast, no impact on ex vivo IgE production was detected in the control group (week 0: 7.0 ± 5.8 ng/mL; week 8: 5.1 ± 5.6 ng/mL; n.s.). By checking the proportions of $CD14^+$, $CD19^+$, $CD4^+$, and $CD8^+$ changes of anti-CD40/IL-4 mediated IgE synthesis due to an alteration of PBMC composition was excluded. However, the total IgE concentration measured in sera was not altered in both groups (data not shown).

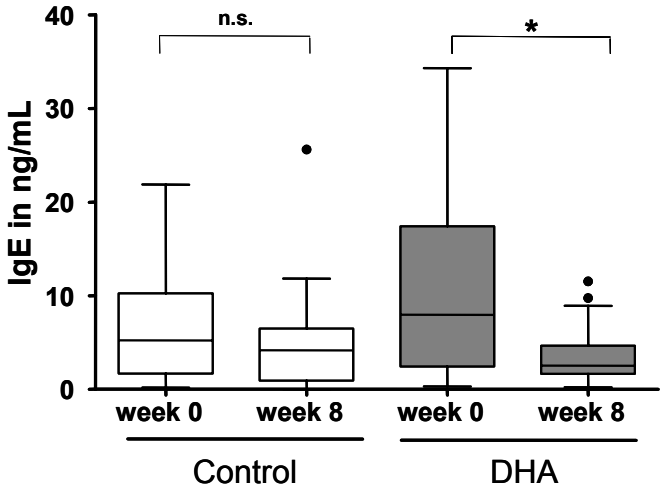

Figure 24: Ex vivo IgE production is inhibited by dietary DHA. IgE production of anti-CD40/IL-4 stimulated PBMC before and after supplementation in the control (n = 23) and DHA group (n = 21). Therefore, isolated PBMC were stimulated with anti-CD40/IL-4 for 10 days. IgE was determined in supernatants by ELISA. Data are shown as box plot (25. and 75. percentile, median); * $p < 0.05$ statistically significant difference compared to baseline values.

3.2.3 Fatty acid supplementation modulates activation of monocytes and B cells

In both groups supplementation led to a significant modulation of the activation status of monocytes ($CD14^+$), but also of B cells ($CD19^+$), as determined by HLA-DR and CD23 expression, respectively. Thereby, proportion of $CD14^+HLA\text{-}DR^+$ and $CD19^+CD23^+$ decreased slightly in relation to the relevant cell population up to week 8. After termination of the supplementation period, proportions increased up to initial values (data not shown).

3.2.4 Systemic IFNγ and IL-4 response remains unaffected by fatty acid supplementation

Additionally, the impact of fatty acid supplementation on systemic cytokine production was investigated. Therefore, untreated or SEB/anti-CD28 stimulated PBMC were analysed regarding IFNγ and IL-4 production of activated $CD69^+$ T_H cells. In unstimulated PBMC a significantly increased percentage of $CD4^+CD69^+IL\text{-}4^+$ was determined whereas the proportion of $CD4^+CD69^+IFN\gamma^+$ decreased at the basal level in both groups during intervention. In contrast, the cytokine production was not altered in SEB/anti-CD28 stimulated PBMC during supplementation. Solely a significant decline of $CD4^+CD69^+IL\text{-}4^+$ was measured in control group (week 0: 0.31 ± 0.14 %; week 8: 0.21 ± 0.12 %; $p = 0.016$). However, no significant differences were detected between both groups (data not shown).

3.3 Oral administration of DHA inhibits the development of eczema in a murine model of protein induced dermatitis

Immunological impact of oral administration of high dose DHA was investigated in a murine model of protein induced eczema. DHA containing diet was well consumed and no side effects were observed. All feeding groups showed a comparable development of body mass, liver and spleen weight.

3.3.1 Dietary DHA reduces the clinical symptoms of protein induced dermatitis

As shown in Figure 25, OVA patched mice developed local dermatitis with macroscopic clinical symptoms, like dryness, erythema, edema, papules, oozing and crusts. Thereby initial systemic sensitisation was essential to elicit visible skin lesions in a significant response rate by e.c. OVA applications [85].

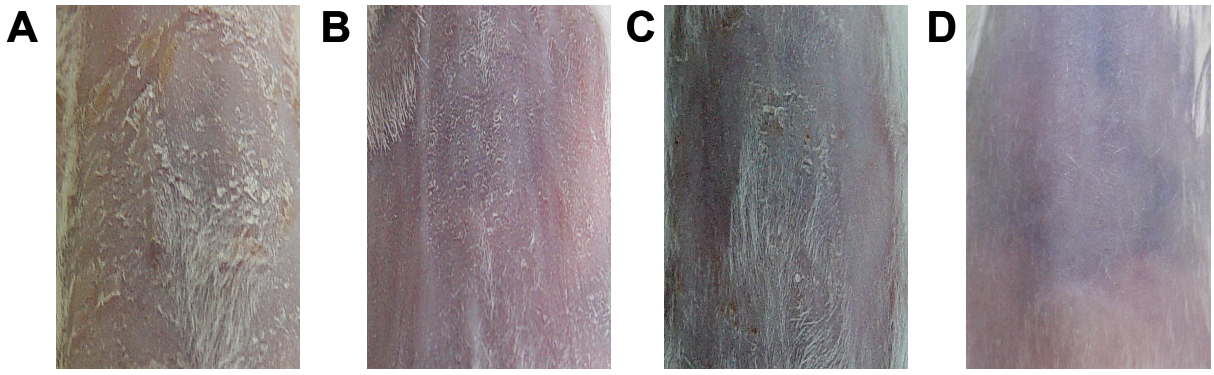

Figure 25: Improved severity of eczema in DHA fed mice. Representative illustration of protein induced dermatitis in mice fed with **A)** control diet, **B)** 2 % DHA and **C)** 4 % DHA in the diet. Picture **D)** illustrates the respective skin area of a non-sensitised control mouse.

As graded by total CSS, e.c. OVA treatment strengthened clinical symptoms from a median of 4 [2 – 5] points in control patched mice (PBS/alum) up to 12 [5 – 12] points in OVA patched mice ($p = 0.017$) (Figure 26). In contrast non-sensitised mice revealed no skin lesions. Following DHA supplementation a significant reduction of CSS was determined compared to control fed diet. Interestingly, 2 % DHA supplemented diet was most effective to reduce the CSS to 7 [5 – 8] ($p = 0.008$), while in 4 % DHA group the outcome was ameliorated to 8 [7 - 10] ($p = 0.032$).

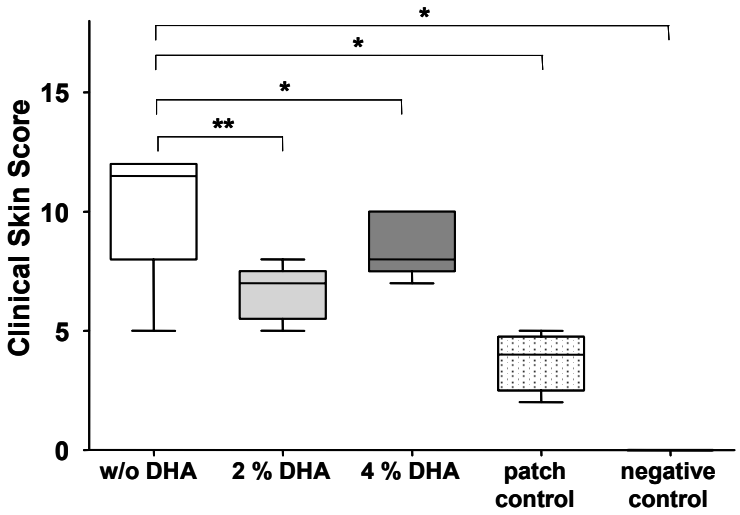

Figure 26: **Oral DHA administration reduces the clinical symptom score of protein induced eczema.** Data are shown as box plot (25. and 75. percentile, median); $n \geq 5$; * $p < 0.05$, ** $p < 0.01$ statistically significant difference compared control fed, OVA sensitised mice.

3.3.2 Dietary DHA reduces the number of $CD8^+$ T cells in eczematous skin

In this murine model of protein induced dermatitis affected skin areas reveal histopathological features of eczema, such as epidermal thickening, parakeratosis, spongiosis and a dense perivascular infiltrate, accompanied by increased MC infiltration and degranulation. Immunohistochemical analysis demonstrates significant increase of dermal $CD4^+$ and of $CD8^+$ cells.

The epidermal thickness was measured eight times per mouse and calculated as average value. In all feeding groups the same range of epidermal thickness was indicated, i.e. no impact of DHA supplementation on this histological feature was assessed (data not shown).

Cellular infiltrates were determined by counting immunohistochemically stained cells in eight sections (100 x 200 µm) per mouse.

Toluidine blue stained MC were located in the dermis and subcutaneous tissue. The number of MC was almost equal in skin preparations of control fed mice and in DHA treated mice (data not shown).

$CD4^+$ cells were located in the dermis, but not in the epidermis. Under DHA treatment no significant alteration in number of infiltrated $CD4^+$ cell was determined (data not shown).

In comparison to $CD4^+$ cells, only a few $CD8^+$ cells were found in the dermis, but also in the epidermis. As depicted in Figure 27, less $CD8^+$ cells (1.7 ± 0.5, $p = 0.032$) were detected in

dermis of 2 % DHA supplemented mice compared to sections of control fed mice (3.9 ± 2.1). Additionally, 4 % DHA supplementation tended to result in a reduced dermal $CD8^+$ number (1.5 ± 1.0, n.s.), but failed to reach significance.

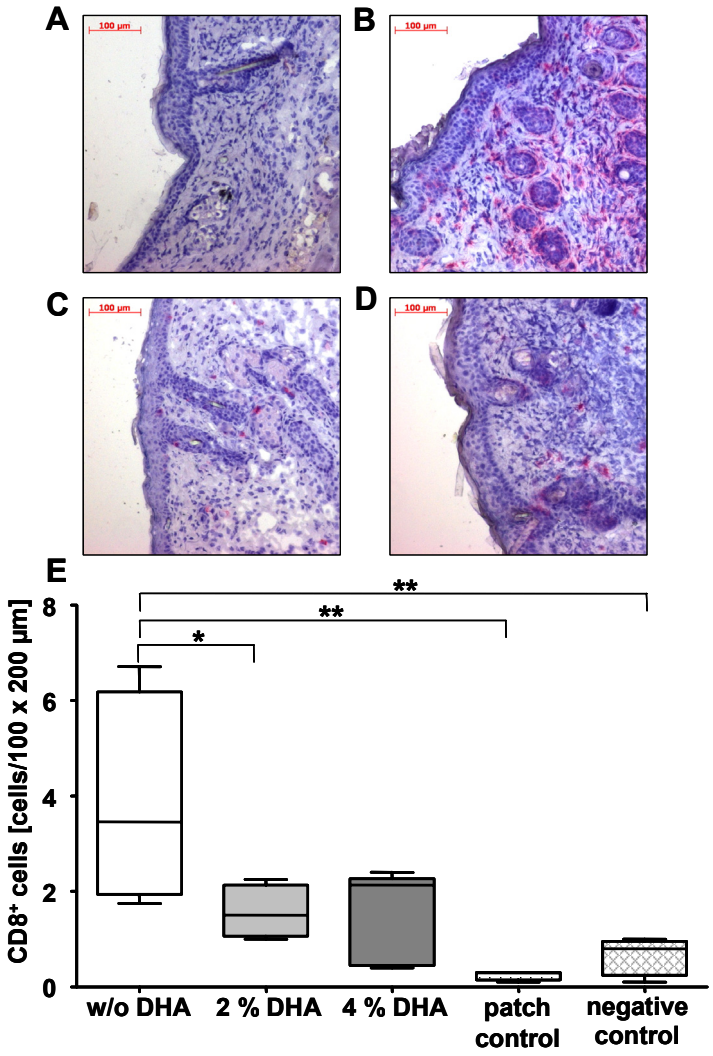

Figure 27: Oral DHA administration reduces the number of $CD8^+$ T cells in eczematous skin. Representative histological image of anti-$CD8^+$ stained 5 µm sections of frozen skin from **A)** a PBS patched BALB/c mouse, from mice with protein induced dermatitis fed with **B)** control diet, **C)** 2 % DHA and **D)** 4 % DHA in the diet. **E)** Positive immunohistochemical stained cells were counted in 100 µm x 200 µm areas at x100 magnification using digital measuring tools. Data are depicted as box plot (25. and 75. percentile, median); $n \geq 5$; * $p < 0.05$, ** $p < 0.01$ statistically significant difference compared control fed, OVA sensitised mice.

3.3.3 Oral DHA administration did not alter the systemic immune response

To evaluate the immunomodulating efficacy of dietary DHA on the systemic immune response, immunoglobulin concentrations were measured in sera by ELISA technique. Nevertheless, no significant impact of DHA treatment on total IgE, OVA specific IgE, IgG_1 and IgG_{2a} serum concentrations was determined (Table 4).

Treatment	w/o DHA	2 % DHA	4 % DHA	negative control
Total IgE [ng/mL]	2384 ± 1059	2427 ± 737	2793 ± 859	59 ± 48 (p = 0.024)
OVA specific IgE [LU/mL]	131 ± 30	144 ± 25	158 ± 32	n.d. (p = 0.031)
OVA specific IgG_1 [LU/mL]	6226 ± 1983	6460 ± 528	6899 ± 2109	n.d. (p = 0.031)
OVA specific IgG_{2a} [LU/mL]	1284 ± 894	871 ± 942	1161 ± 1276	n.d. (p = 0.031)

Table 4: Oral DHA administration does not affect systemic immune response. Total IgE**,** OVA specific IgG_1, IgE and IgG_{2a} concentrations were analysed in sera of OVA sensitised and OVA sensitised plus DHA fed mice by ELISA. Data represent mean ± SD; in brackets: p as compared to control fed, OVA sensitised mice; negative control – non-sensitised mice; n.d. - not detectable.

4 Discussion

The incidence of common allergic diseases has increased quite dramatically during the last decades and in many countries the prevalence has reached almost epidemic proportions [1]. At this time a cure for these diseases does not really exist, although a wide range of treatments are used to control the symptoms. The costs are manifold including both, direct and indirect components [23]. Thus, allergies have become a major medical issue of the Western world. Since there is an indication that the nutritional factors, especially the changing dietary fatty acids may contribute to this alarming development, many investigations have been done in this field [74,75].

The first evidence for relevance of dietary n-3 PUFA in inflammation was derived from epidemiological observations displaying a low incidence of autoimmune and inflammatory disorders in a population of Greenland Inuit compared with gender and age matched groups living in Denmark [86]. Most of these diseases are designated by inappropriate activation of T cells and destruction of tissues. Interestingly, native Greenland Inuit [87] and Japanese [88] consume high amounts of long chain n-3 PUFA from seafood and have a low incidence of chronic inflammatory or autoimmune disorders, even compared to their Westernised ethnic counterparts.

It is very likely that DHA and EPA are responsible for the beneficial effects of fish oils on the immune system [53,89]. Until now the majority of studies dealing with the immunomodulatory effects of long chain n-3 PUFA have been dealing with fish oils, what limits the understanding of the particular effects of EPA and DHA and prohibits their comparison. There are still controversial results regarding which n-3 PUFA is accountable for the effects of fish oil [90,91,92]. Thereby, DHA exerts a lot of favourable effects in many diseases and is known to be antiinflammatory. However, some researchers assume that DHA is the biologically active component of fish oil [93].

4.1 DHA inhibits IgE production in human B cells

Allergic individuals exposed to minute amounts of allergen experience an immediate immune response with possibly a fatal outcome. The spreading epidemic of allergies and atopic diseases has generated a great interest in IgE, the central molecule of type I allergic reactions. IgE is usually found at low concentrations in the plasma and mainly secreted by plasma cells in mucosa associated lymphoid tissue. Patients suffering from atopic disorders, like asthma, allergic rhinitis and extrinsic atopic eczema have elevated serum levels of both, total IgE and allergen specific IgE. The differentiation of a B cell to an IgE producing cell is tightly adjusted

via CSR [5,94]. Consequently, a potential target for prevention and treatment of atopic diseases by DHA is the regulation of IgE production, in particullar the switch towards IgE.

To induce IgE synthesis in vitro, cells were stimulated with anti-CD40 and IL-4 [80]. Application of DHA resulted in a dose-dependent inhibition of IgE production in both, PBMC and B cells. This effect was isotype specific since both, IgG and IgA remained unchanged. Toxic or antiproliferative effects were excluded using B cells labelled with CFSE and WST-1 assay as well as by staining with tryptan blue and propidium iodide for identification of dead cells.

Furthermore, it was investigated if the reduced amount of IgE in supernatants was due to inhibition of immunoglobulin secretion, altered differentiation into IgE secreting plasma cells or a result of impaired switching towards IgE.

DHA dose-dependently inhibited the anti-CD40/IL-4 driven differentiation to IgE secreting cells. However, the number of IgA secreting cells was not altered, excluding the possibility of a general inhibited immunoglobulin secretion and indicating IgE specific alteration of plasma cell differentiation.

It was determined that DHA specifically interfered with the IgE switching process at the transcriptional level of AID and εGLT. The importance of AID in CSR got obvious after rigorous impairment in $AID^{-/-}$ mice [95] as well as in patients with autosomal recessive hyper-IgM syndrome (HIGM2), who carry mutations in the AID gene [96]. However, DHA inhibited AID mRNA expression dose-dependently suggesting interference in AID dependent switch recombination. Interestingly, IgA and IgG remained almost unchanged regarding production and number of antibody secreting cells indicating that the dimension of DHA mediated AID inhibition was not sufficient to affect switch recombination in general.

CSR towards IgE in B cells stimulated with anti-CD40/IL-4 involves a number of molecular events [97]. The transcription of εGLT precedes B cell IgE synthesis and the dependency of switching to IgE on its expression has been reported [83]. Consequently, the dose-dependent inhibition of εGLT points to the IgE specific action of DHA. Thereby, DHA caused this obvious inhibition of IgE by reducing εGLT transcription together with decreasing expression of AID.

B cell IgE production is regulated by cytokines like IL-4 [98] as well as direct contact between B and T cells [99], especially by CD40/CD154 interaction [80]. Importantly, AID [100] and εGLT [101] are dependent on IL-4 and CD40, whose downstream signalling is mediated through STAT6 and NFκB. Since DHA downregulated εGLT expression in B cells stimulated

with anti-CD40 or IL-4 alone, the involved signalling pathways were examined by analysis of STAT6 phosphorylation and NFκB p50 translocation into nucleus.

Preincubation of B cells with DHA caused a dose-dependent inhibition of STAT6 phosphorylation. The analysis of the total STAT6 protein revealed no impact of DHA, but a decreased pSTAT6/STAT6 ratio. These data exclude the decrease of STAT6 protein expression by overnight incubation with DHA and point to its exclusive direct interference in IL-4 signal transduction. These data are in line with previous reports by Gorjao et al. [102]. They detected a decreased IL-2 mediated JAK1 and JAK3 phosphorylation and consequently diminished STAT5 phosphorylation through DHA. This was associated with a reduced surface expression of the IL-2 receptor resulting from an altered lipid raft organisation.

Other studies confirmed that DHA has properties to interfere with lipid raft and membrane domains [62,68]. Stillwell et al. [103] recently described that dietary consumption of DHA creates DHA-rich membrane domains which lead to dislocation of sterol into sphingomyelin-rich lipid raft domains. In these environments the acyl chain order is extremely different. Thereby, protein movement between the domains is associated with a change of conformation and consequently has the potential to modulate cellular signalling. It has also been reported that DHA treatment causes stimulation of PLD1 activity in human PBMC by partial disorganisation of membrane microdomains, which results in activating the protein and disrupting signal transduction events [104]. Such mechanisms may apply for DHA action on STAT6 phosphorylation. This assumption is supported by the fact that DHA preincubation was necessary to reduce the IL-4 driven STAT6 phosphorylation possibly via downregulation of IL-4R expression.

Even though, IL-4 plays a key role in the production of IgE by B cells [105] and provokes εGLT, IL-4 alone is not sufficient to induce IgE production. However, additionally to IL-4, CD40 signalling is indispensable for induction of the mature ε transcripts and consequent IgE synthesis [80,106,107].

CD40 ligation, along with the other B cell stimuli, results in activation of transcription factors belonging to the Rel/NFκB family [13,14]. It is reported that genes involved in isotype switching like AID are regulated by type 1 but not type 2 NFκB activity [15]. Transcriptional regulation by type 1 NFκB involves activation of IKK complex leading to degradation of IκBα and IκBβ and as a result release NFκB p50:p65 and p50:cRel dimers [108]. Additionally, mice lacking p50 show a clear reduction of IgE indicating the central role of p50 in IgE production [109]. Therefore, the impact of DHA on degradation of IκBα and nuclear translocation of p50

was investigated thoroughly. DHA reduced the anti-CD40 driven translocation of p50 into the nucleus, thereby affecting the second essential signal for IgE switch, IgE plasma cell development and IgE production. These data are in line with previous reports showing that DHA inhibits NFκB activation [110,111]. Interestingly, DHA did not affect IκBα degradation which is an important upstream event for p50 release. However, NFκB can dissociate from phosphorylated IκBα and move into the nucleus without IκBα degradation, indicating that a reduced p50 translocation is not necessarily related to reduced IκB degradation [112].

Another essential step for p50 translocation is its generation by processing from the precursor p105. This sequence is initiated by ubiquitinylation, followed by gradual proteasomal degradation of the p105 from the C-terminal end [15]. DHA acts probably via modulation of ubiquitinylation [15] or alteration of proteasomal activity [113], consequently resulting in altered p50 translocation and thus modulated transcriptional activity.

DHA inhibited the anti-CD40/IL-4 driven IgE production in PBMC of healthy individuals and allergic donors with lower serum IgE, but had no impact on PBMC of atopic patients with high serum IgE. Previous reports show that IgE hyperproduction in patients with atopic eczema is determined by constitutive and enhanced JAK3 phosphorylation in B cells [114]. This could explain the missing effect of DHA in patients with high serum IgE. The increased inherited JAK3 activation prevents the modulation of the IL-4 signalling pathway by DHA.

DHA leads to a profound repression of the IgE switching process and thus IgE plasma cell development as well as IgE production in human B cells. This is caused by an early inhibition of CD40 and IL-4 signalling pathways resulting from a simultaneously decreased STAT6 and NFκB activation [115]. Therefore, the slight reduction of IgG secreting cells after treatment with 10 µM DHA may result from this concomitant inhibition, since CD40 ligation and IL-4 induce not only IgE, but also IgG_4 [107,116]. Hereby, DHA acted selectively on IgE isotype by means of immune modulation rather than suppression. These results comprise a strong biological relevance for the development of allergies, since it has been shown that the DHA concentrations used in this study correspond to serum levels found in humans [117]. More importantly, upon high dose DHA supplementation in patients with extrinsic atopic eczema the inhibition the anti-CD40/IL-4 driven IgE production in allergic PBMC can be also seen ex vivo [84].

In terms of IgE production and its related signal transduction, these data reveal the early interference of DHA in a possibly longer chain of events initiated by DHA. It cannot be excluded that DHA causes additional effects on IgE producing B cells later on. Thereby, modu-

lation of eicosanoid synthesis, nuclear receptor binding and activation / repression, regulation of gene expression, alteration of cytokine production etc. may be potential effects provoked by DHA. Certainly, these observations might be important for dietary management of IgE mediated diseases and may offer an opportunity for a successful prevention of allergic diseases.

4.2 DHA supplementation in atopic eczema – a randomised, double blind, controlled study

Several clinical trials have been conducted to ameliorate symptoms of atopic eczema by supplementation with n-6 PUFA. Based on the data generated so far, there is a lack of evidence supporting the use of n-6 fatty acid supplementation on improvement the clinical outcome of atopic eczema [30]. Due to its known antiinflammatory effects, some clinical trials have been also focused on fish oil supplementation [118,119]. Particularly, the beneficial efficiency of DHA supplementation in vivo has been previously suggested by several studies [63].

The assumption that DHA may be therapeutically effective in atopic eczema is based on the prerequisites that atopic eczema is an inflammatory disease which is amplified by an excessive production of proinflammatory eicosanoids [120,121,122] and is associated with a abnormal fatty acid metabolism [28], that DHA supplementation acts antiinflammatorily (among others) by modulating eicosanoid generation [53] and there is a correlation between intake and a decreased risk of atopic eczema [123,124]. However, there were no accessible reports on clinical trial with dietary highly purified DHA in atopic eczema. Accordingly, a randomised, double blind, controlled trial was performed to evaluate the therapeutical impact of 8 week supplementation with 5.4 g of highly purified DHA daily in this atopic skin disorder. Thereby, DHA, but not the control treatment resulted in a significant clinical improvement of atopic eczema in terms of a decreased SCORAD. A significant reduction of anti-CD40/IL-4 mediated IgE synthesis of PBMC was detected in DHA group only. Concomitantly, DHA treated patients showed an increase of plasma n-3 PUFA and a decrease of n-6/n-3 PUFA ratio. Finally, supplementation led to a modulated activation status of PBMC in both groups [84]. However, there was no significant difference between DHA and control group. Thus, the possibility of a placebo effect cannot be excluded, despite the fact that DHA and control capsules were indistinguishable. Furthermore, the small study size limited the statistical power of these comparisons. Due to the lack of reported data showing the clinical efficacy of dietary DHA in atopic eczema the exact calculation of the necessary number of patients prior to this pilot study was not possible. Therefore, this exploratory trial with an arbitrary sample size was

performed. This limitation might have led to the possibility that randomisation was not likely to ensure the desired prognostic comparability between DHA and control group. However, both treated groups were well matched for gender, age and BMI and the study was double blinded. To determine whether the observed clinical effect of dietary DHA is of therapeutical significance needs further clarification and confirmation.

As already demonstrated in former studies [125], an increased plasma n-3 PUFA proportion was detected in all patients consuming DHA [84]. Due to DHA treatment plasma fraction of DHA and EPA increased two- to threefold indicating an excellent compliance. The elevated EPA plasma level may be explained either by retroconversion of DHA to EPA when used in high doses [126] or the amount of EPA (0.4 g/d) contained in the used capsules or both. The detected clinical efficacy in therapy of atopic eczema points to antiinflammatory properties of DHA. Metabolism via COX and LOX pathways is highly dependent on the availability of lipid precursors [127,128]. Indeed, DHA does not directly act as a substrate for eicosanoid synthesising enzymes, but inhibits AA metabolism [66] and serves as a precursor of n-3 eicosanoids via EPA [126]. DHA is known to reduce PGE_2 production [66] and expression of PG synthesising enzyme COX-2 [68]. Accordingly, a favourable shift in eicosanoid formation may be responsible for several effects of DHA supplementation. In addition, DHA, possibly in combination with EPA, can act as precursor for COX and LOX driven bioactive lipids such as lipoxins, protectins and resolvins which are known to be antiinflammatory [129]. Importantly, an alteration of PUFA level following allergic sensitisation [130] as well as an involvement of n-6 PUFA derived mediators in the pathophysiology of atopic eczema is suggested since patients have an enhanced synthesis of these metabolites and thus increased concentrations of AA derived LT and PG in skin lesions [120,121]. Mayser et al. [32] demonstrated that daily infusions of n-3 PUFA effectively ameliorate the clinical outcome of atopic eczema. This therapeutical effect was accompanied by alterations in fatty acid profile of membrane lipid pool as well as changes in eicosanoid synthesis. Following the rise of free EPA an increase of n-3 PUFA derived metabolites was observed, which may also be associated with the clinical improvement. Furthermore, it is speculated that a combination with dietary supplements is capable to enhance this therapeutical effect. However, patients of the DHA group showed a strong reduction of plasma n-6/n-3 PUFA ratio and a significant clinical improvement. It is likely that the fatty acid pattern in the skin was also altered in a similar way, since it has been shown that dietary n-3 PUFA supplementation is associated with their increased incorporation into skin [131]. EPA and DHA play an important role for stratum corneum and are therefore essential for maintaining a normal function and structure of skin barrier [132]. Additionally, it is discussed that components of the lipid metabolism pathway are involved in

pathogenesis of atopic eczema and represent an opportunity to manage and perhaps even prevent subsequent atopic disorders through restorement of the barrier [133,134]. Willemsen et al. have recently shown that DHA effectively supports skin barrier integrity by improving resistance and permeability [135]. Lipid infusions may result in sufficient availability of fatty acids in skin. Whether this can also be achieved by oral supplementation needs to be investigated in further investigations.

Extrinsic atopic eczema is associated with an unbalanced cytokine pattern promoting an IgE response to common environmental allergens [24]. In this study a reduced ex vivo anti-CD40/IL-4 mediated IgE synthesis by DHA supplementation was determined [84]. These data are in line with the B cell in vitro results showing the DHA mediated inhibition of anti-CD40/IL-4 driven IgE production comprising a strong biological relevance regarding the development of allergies.

Since a change of total fat intake influences several immunological parameters [118] the control supplement contained the same amount of fat as the DHA capsules. Short to middle chained saturated fatty acids exhibit a low biological efficacy [136]. Hence, the control capsules supplied the middle chained fatty acids, caprylic and capric acid. Therefore, the effects caused by an increased amount of dietary fat were intended to be eliminated.

Analysis of the systemic IL-4 and IFNγ response revealed no impact by fatty acid supplementation suggesting that DHA acts rather locally than on systemic level. The determined non-specific increased IL-4 with a concomitant decreased IFNγ may be attributed to seasonal influences resulting from natural exposure to allergens in spring [137]. However, the reduced IL-4 levels in control group suggest a potential immunological activity of the control supplement. This is supported by the finding, that both, DHA and control supplementation led to significantly lower HLA-DR surface expression on monocytes and CD23 expression on B cells. This fact points to a requirement of a three-armed, placebo controlled study in order to compare DHA, an isoenergetic fatty acid control supplement and a placebo without any fat. Nevertheless, the unchanged anti-CD40/IL-4 stimulated IgE production within the control group suggests DHA specific pathways.

In conclusion, although the sample size may be underpowered to detect small but relevant effects, the clinical results imply that dietary DHA may be beneficial for the standard treatment of atopic eczema. It may not be excluded that different doses of DHA and long term treatment are more effective in atopic eczema. To deal with this subject, an extensive randomised, placebo controlled, three-armed follow up trial should be performed.

4.3 Oral administration of DHA inhibits the development of eczema in a murine model of protein induced dermatitis

A mouse model of allergen mediated dermatitis triggered by e.c. application of proteins was initially described by Wang et al. [44] and Spergel et al. [45]. To investigate the impact of oral DHA administration on pathogenesis of protein mediated dermatitis, this existing model was improved to achieve visible lesions. Thereby, the typical macroscopic skin characteristics as well as high response rates were evoked by initial systemic sensitisations implicating the importance of interactions between the local and systemic immune responses for clinical manifestation [85]. After all, OVA sensitised mice developed local inflammatory skin lesions which were clinically, histologically and immunologically comparable to human atopic eczema. Lesional skin areas exhibited significant histological characteristics such as skin thickening, increased cellular infiltrates containing $CD4^+$, $CD8^+$ T cells and MC as well as induced lesional expression of T_H2 cytokines (IL-4, IL-10). These observations are consistent with reports on acute skin lesions in humans [26]. Additionally, elevated allergen specific IgE and IgG was detected in the serum of these mice [85].

This small exploratory study was aimed to investigate the clinical efficacy of oral DHA administration in a murine model of protein mediated dermatitis and to research into the underlying systemic and in particular local mechanisms of DHA effectiveness. Since there are only few data regarding supplementation of pure or highly enriched DHA in mice in general and no accessible data concerning protein induced eczema, this pilot study was performed with an arbitrary sample size. It is likely, that the small study size limited the statistical power and might have caused a failing of statistical significance comparing DHA and control group.

In terms of skin disorders, merely Tomobe et al. [93] investigated the effects of 4.8 % DHA of total diet on the inflammatory response of contact hypersensitivity reactions. Since they used an extremely high dosage, the therapeutical range of DHA application had to be delineated in this study. Thereby, the applied DHA dose should have had an approximated dosage which is achievable by dietary intake in humans. Otherwise, humans and mice do not appear to be equally responsive to dietary n-3 PUFA, although a comparable pattern of changes in immune cell PUFA content following supplementation was detected. It is known that the murine cell membranes contain initially more DHA and become more enriched with DHA relative to humans at comparable levels of DHA in the diet [128]. Consequently, a direct inference from humans to mice or vice versa is complicated. Nevertheless, this pilot study was performed to reveal the clinical efficacy of 2 % and 4 % DHA.

In this current investigation DHA supplementation led to an improved clinical outcome in a murine model of allergen induced dermatitis. The DHA mediated decrease of CSS was associated with a reduced number of dermal $CD8^+$ T cells. Thereby, supplementation with 2 % DHA in the diet was more potent to improve the clinical outcome of allergen induced dermatitis than 4 % DHA application.

As discussed for human supplementation trial, DHA may mediate some of its favourable effects through changing the production of immunomodulatory lipid mediators and thus ameliorating the outcome of protein induced dermatitis [91,130,138].

A mediator related effect of DHA may be associated with eicosanoid derived properties. This is supported by publications showing that amelioration of inflammatory and allergic symptoms by n-3 PUFA ingestion has been connected to reduction in 4-series and an increase in 5-series of LT [139,140]. Interestingly, supplementation with 2 % DHA in the diet was more clinically efficient compared to 4 % DHA. Broughton et al. [141] have demonstrated that eicosanoid production in vivo is dependent on both, the n-3/n-6 ratio and the amount of total fat. High levels of dietary fat reduced 5-series LT synthesis simultaneously favouring synthesis of 4-series LT in mice fed with diets containing high n-3/n-6 ratios. Thus, high fat intake may offset some benefits of n-3 PUFA supplementation. This immunological effect by increased total fat intake can be enhanced when DHA membrane content has reached the plateau [128]. Furthermore, it has to be considered that both DHA diets were prepared with different DHA blends containing additional fatty acids in varying concentrations. The mixtures might have influenced the outcome in both DHA supplementation groups respectively.

DHA improved the clinical outcome of eczema but did not affect serum immunoglobulin concentration as determined in the human clinical study with DHA capsules as well [84]. Furthermore, flow cytometric analysis of systemic cytokine response indicates no impact by oral DHA administration (data not shown). These data suggest that DHA operates rather on the local sites of inflammation than on the systemic immune response. Otherwise, the in vitro data show an inhibition of the IgE switching process. The question arises why DHA did not modulate the IgE response despite these in vitro findings. Probably, DHA supplementation was not capable to inhibit the in vivo IgE switching process since parallel DHA treatment and OVA sensitisation did not permit the sufficient DHA membrane loading [128]. Possibly, a preventive application of dietary DHA preceding the initial sensitisation may provoke this DHA mediated feature in vivo as well.

T cells dominate the cellular infiltrate in eczematous skin lesions. Thereby, immigrating $CD4^+$ and $CD8^+$ T cells are shown be allergen specific [142,143,144]. DHA treatment did not alter

dermal infiltration of $CD4^+$ T cells, but significantly reduced the number of dermal $CD8^+$ cells. This finding was more prominent in mice fed with 2 % DHA compared to animals' receiving 4 % DHA. The role of epidermal $CD8^+$ cells in pathogenesis of atopic eczema has been already suggested [142,145]. $CD8^+$ T cells depleted mice fail to develop significant signs of inflammation upon allergen exposure, whereas $CD4^+$ cell depletion does not prevent protein mediated eczema [146] suggesting $CD8^+$ T cells as important effector cells in allergen induced skin inflammation. Whether DHA causes this local reduction of $CD8^+$ cells by increased apoptosis [147,148] or decreased recruitment into skin will need further verification [149].

Differential expression of ligands and their receptors allows tissue specific migration of T cells [148,150]. CLA is a marker for cells migrating into skin. CLA^+ T cells are involved in pathogenesis of extrinsic atopic eczema and display a distinct expression profile of proteins involved in activation, differentiation, migration and apoptosis [148]. Skin homing $CD8^+$ T cells are known to induce keratinocyte apoptosis, an important mechanism for induction and maintenance of eczema [151,152]. Importantly, responsiveness to oral n-3 PUFA administration for circulating immune cells is not the same as for tissue immune cells within the same lineage [128]. Thus, DHA may affect T cells in the skin in a different mode than blood T cells.

Local production of chemotractant chemokines and the expression of their corresponding receptors on the cell surface are also involved in regulation of T cell migration [19]. CCL27 is a skin specific chemokine exclusively produced by epidermal keratinocytes and attracts CCR4 expressing T_H2 cells into skin [153]. A modulation of the lesional chemokine production by DHA thereby reducing the number of attracted $CD8^+$ cells may be a possibility.

Additionally, the interaction of keratinocytes with infiltrating T cells plays an essential role in pathology of atopic eczema [154]. Interestingly, keratinocytes become activated by interaction with T cells through CD40/CD40L [154]. DHA inhibits CD40 dependent nuclear p50 translocation in human B cells. Whether DHA operates in an analogous manner in mice has to be elucidated. However, own unpublished data reveal a prominent DHA mediated inhibition of IgE production in cultures of murine B cells upon anti-CD40/IL-4/LPS stimulation (data not shown). Potentially, CD40 signalling pathway in murine keratinocytes may be also targeted and thus modulated by DHA. Keratinocytes release T cell attracting chemokines and influence T cells by their secreted cytokines hence contributing to disease progress [154]. Reduced keratinocyte activation might be partly responsible for DHA mediated amelioration

of allergen induced dermatitis. In vitro it has been already shown that DHA is capable to decrease production of TNFα and IL-8 in keratinocytes [155].

Nuclear hormone receptors, like RXR [156], LXR [157] and PPAR [149] are known to be involved in skin homeostasis. Furthermore, their ligands modulate allergic immune response at the molecular level [158,159,160]. Therefore, agents stimulating these ligand activated transcription factors are discussed as potential therapeutical target in combating atopic eczema [157].

Mice lacking RXRα/β in their epidermal keratinocytes display a chronic dermatitis mimicking that observed in atopic eczema patients. Furthermore, they exhibit a systemic syndrome similar to that found in these patients [161]. DHA has been demonstrated to bind to the RXR ligand binding domain [73,162], to recruit coactivator peptides [163] and to activate this transcription factor [162,164]. Modulation of RXR activity can influence a broad spectrum of genes, since RXR acts as heterodimer with other nuclear receptors on transactivating transcription [165].

PPAR have been identified in keratinocytes and play an important role in skin barrier function and regulation of skin inflammatory response [149,166]. PPARα deficient mice show increased epidermal thickening, dermal recruitment of inflammatory cells in addition to increased IgE and IgG_{2a} production. In these mice inflammation was correlated to an unbalanced cytokine response and an increased expression of NFκB in the skin. PPAR ligands inhibit the secretion of chemokines thereby decreasing leukocyte recruitment to sites of inflammation [167]. Interestingly, PPARα expression was decreased in eczematous skin from atopic eczema patients, suggesting that defective PPARα expression might contribute to the pathology of this inflammatory skin disorder [168]. Consequently, PPAR activating molecules may regulate skin homoeostasis and may have therapeutical implications [149]. In this context, DHA [164,169,170] and some of its derivates [171] are already shown to activate PPAR and so transcribing various PPAR dependent genes.

Further on, LXR is also essential for epidermal permeability barrier homeostasis, since activation stimulates keratinocyte differentiation, lipid synthesis and increases lamellar bodies formation / secretion [166]. It is also reported that activators of LXRα and LXRβ exhibit potent antiinflammatory activity in models of allergic contact dermatitis [172]. Interestingly, there are evidences which suggest that DHA modulates LXR dependent gene expression [173,174].

Taken together, dietary DHA improves the clinical outcome of allergen induced dermatitis in mice by modulating the local immune response. DHA supplementation reduces the number

of dermal $CD8^+$ T cells which are essentially involved in pathogenesis of murine dermatitis. The related mechanisms, like local prostanoid production, cytokine and chemokine expression as well as activation status of involved cell types have to be investigated. Additionally, further examinations are necessary for determination of optimal time point and dosage of DHA application for gaining maximum of information about the local processes in skin under DHA treatment.

4.4 Conclusion

Within the scope of a changing life style as well as altering dietary habits epidemiological and experimental data suggest a causal relation between the decreasing n-3 PUFA intake and the increasing prevalence of atopic diseases. Although complete prevention of atopic eczema and other atopic disorders seems to be not feasible, interventions which modify disease expression may be possible. In order to improve allergy therapy the potential benefits of dietary DHA as well as its underlying mechanisms were investigated in vitro, in a mouse model of protein induced dermatitis and in a randomised clinical trial with atopic eczema patients.

The in vitro investigation revealed that DHA acts directly on B cells leading to a profound repression of the IgE switching process. Consequently, IgE plasma cell development and IgE production in human B cells is inhibited. This is caused by an early inhibition of CD40 and IL-4 signalling pathways resulting in a simultaneously decreased STAT6 and NFκB activation.

Unanswered is still the question whether DHA is simply incorporated into phospholipids and exerts its effects through modulation of membrane properties or does DHA penetrates into cell and potentially binds to a specific receptor. Subcellular membrane fractionation of cells after incubation with radionucleotide labelled DHA may give information about the localisation of DHA in the cell.

DHA inhibited the expression of εGLT in anti-CD40/IL-4 stimulated B cells. Does DHA also act on transcription of other germline transcripts like γGLT and αGLT?

Furthermore, the data show that DHA application results in a reduced STAT6 phosphorylation. Whether this DHA mediated diminishment of IL-4 signalling is associated with a reduced JAK1 and JAK3 activation and / or an impairment of surface IL-4R complex expression has to be elucidated.

DHA reduces the anti-CD40 driven nuclear NFκB p50 translocation without affecting IκBα degradation. The question arises whether DHA targets the liberation of p50 or does DHA

prevent the entry into nucleus. Are other members of the Rel/NFκB family affected as well? Analysis of IKK phosphorylation and investigation of cytosolic p105 may help to understand the basic mechanisms.

Supplementation with 5.4 g/d highly purified DHA over 8 weeks resulted in a significant clinical improvement of atopic eczema in this randomised, double blind, controlled trial. Additionally, a significant reduction of anti-CD40/IL-4 mediated IgE synthesis of PBMC was detected in the DHA group only. These effects were associated with an increase of plasma n-3 PUFA and a decrease of n-6/n-3 PUFA ratio. However, supplementation did not affect the systemic cytokine production, but led to a modulated activation status of PBMC in both groups.

Dietary DHA administration has directed to an impressive modulation of the plasma fatty acid spectrum. Whether this modification reflects the PUFA incorporation into the phospholipids of immune cells or particularly into membranes in skin lesions has to be elucidated. Furthermore, it has to be investigated whether the altered fatty acid profile leads to a favourable manipulation of eicosanoid production both locally and systemically.

In the DHA group ex vivo anti-CD40/IL-4 mediated IgE synthesis was significantly inhibited. These findings illustrate the strong biological relevance of the in vitro data. Whether this inhibition is caused by a diminished STAT6 and NFκB signalling in B cell can be discovered by analysis of B cells from DHA supplemented donors.

The reduced activation status of PBMC in both groups implies a biological active control supplement. Not only the quality, but also the quantity of lipids influences immunological parameters. This fact points to a requirement of a three-armed, placebo controlled study in order to compare DHA, an isoenergetic fatty acid control supplement and a placebo without any fat. Thereby, the basic diet of participants might be defined and standardised to ensure comparable premises. Additionally, study of local processes in lesional skin after DHA supplementation should be performed. This includes histological characterisation of cellular infiltrates ($CD4^+$, $CD8^+$, MC, DC), transcriptional analysis of local chemokines expression pattern (e.g. CCL27) and investigation of local PUFA phospholipid content as well as fatty acid mediator profile.

In the last part of this thesis, the clinical efficiency of oral DHA administration was verified in a mouse model of allergen induced eczema thereby investigating local processes in eczematous skin. DHA supplementation caused an improved clinical outcome of protein induced dermatitis. The DHA mediated amelioration was associated with a reduced number of dermal

$CD8^+$ T cells. Interestingly, supplementation with 2 % DHA was more potent to improve the clinical outcome of allergen induced dermatitis than 4 % DHA application. However, DHA administration did not affect serum immunoglobulin concentrations.

It is known that DHA administration alters the plasma fatty acid in mice [130]. In order to delineate the local DHA mechanisms leading to an improved CSS, analysis of PUFA membrane content as well as local DHA metabolite generation has to be carried out.

The clinical amelioration by oral DHA was associated with a reduced number of dermal $CD8^+$. Further investigations to reveal the underlying trigger factors may imply the histological examinations of cell specific apoptosis and proliferation as well as keratinocyte activation. Since chemokines are involved in orchestrating cellular skin infiltration, expression analysis may be of importance. Moreover, DHA is a potential ligand of some nuclear receptors, which are described to be aphysiologically expressed in lesional skin of atopic eczema patients. Therefore, histological investigations of e.g. PPARα and NFκB in eczematous skin of DHA treated versus control fed mice might be of interest.

Keratinocytes are involved in pathogenesis of atopic eczema and are known to be activated by T cell interaction through CD40/CD40L. This communication may be affected by DHA. In vitro investigations probably will reveal the direct impact of DHA on keratinocytes. Analysis of CD40 and NFκB expression may be thereby of importance.

Finally, in order to achieve the full spectrum of DHA affects different DHA treatment protocols might be performed. Thereby, a preventive application of dietary DHA preceding the initial sensitisation may help to reach the sufficient DHA membrane loading for inhibition of IgE production in vivo. Potentially, DHA administration to the mothers before delivery or directly to offspring will be an opportunity.

In summary these data show that dietary DHA has the capacity to influence the allergic immune response. These current observations support previous reports suggesting the immunomodulatory impact of this dietary component. Referring to this for achieving a selective manipulation of allergy it is necessary to promote the understanding of the underlying mechanisms of action of both DHA as well as its involved metabolites.

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Appendix

Antibodies

Antibody	Clone	Manufacturer
Anti-human CD4 PerCP	SK3	BD PharMingen, San Diego, USA
Anti-human CD14 PE	MϕP9	BD PharMingen, San Diego, USA
Anti-human CD19	Magnetic beads	Miltenyi Biotec, Bergisch-Gladbach, Germany
Anti-human CD19 FITC	4G7	BD PharMingen, San Diego, USA
Anti-human CD20 FITC	2H7	eBioscience, San Diego, USA
Anti-human CD23 APC	EBVCS-5	BD PharMingen, San Diego, USA
Anti-human CD27 FITC	LG.7F9	eBioscience, San Diego, USA
Anti-human CD27 PE	LG.7F9	eBioscience, San Diego, USA
Anti-human CD38 PE Cy5.5	HIT2	eBioscience, San Diego, USA
Anti-human CD69 APC	L78	BD PharMingen, San Diego, USA
Anti-human CD138 APC	MI15	BD PharMingen, San Diego, USA
Anti-human CD40	82111	R&D, Minneapolis, USA
Anti-human CD40	G28.5	DRFZ
Anti-human CD28	L293, L25	BD PharMingen, San Diego, USA
Anti-human HLA-DR PerCP	L243	BD PharMingen, San Diego, USA
Anti-human IFNγ FITC	4S·B3	BD PharMingen, San Diego, USA
Anti-human IgA	G20-359	BD PharMingen, San Diego, USA
Anti-human IgG	G18-145	BD PharMingen, San Diego, USA
Anti-human IgE	HP6061, HP6029	Southern Biotech, Birmingham, USA
Anti-human IgA, IgG, IgM	Matched pairs	Jackson ImmunoResearch, West Grove, USA
Anti-human IκBα Alexa Fluor 647	L35A5	Cell Signalling Technology Inc., Boston, USA
Anti-human IL-4 PE	8D4-8	BD PharMingen, San Diego, USA
Anti-human p50	H119	Santa Cruz, Heidelberg, Germany
Anti-human pSTAT6 Alexa Fluor 647, (pY641)	18	BD PharMingen, San Diego, USA
Anti-human STAT6 PE	23	BD PharMingen, San Diego, USA
Anti-mouse CD4	L3T4	BD PharMingen, San Diego, USA

Anti-mouse CD8	RM4-15	BD PharMingen, San Diego, USA
Anti-mouse IgG1, biotin	A85-1	BD PharMingen, San Diego, USA
Anti-mouse IgG2a, biotin	R19-15	BD PharMingen, San Diego, USA
Anti-rabbit HRP	Goat, Sc-2004	Santa Cruz, Heidelberg, Germany
Anti-rat IgG, biotin	Goat	BD PharMingen, San Diego, USA
Purified mouse IgE, κ	C38-2	BD PharMingen, San Diego, USA

Chemicals

Chemicals / Reagents	Manufacturer
Acryl amide, 30 %	Roth, Karlsruhe, Germany
Albumin from chicken egg white, Gade V	Sigma-Aldrich, Steinheim, Germany
3-Amino-9-ethyl-carbazole (AEC)	Sigma-Aldrich, Steinheim, Germany
Ammunium persulfat (APS)	Sigma-Aldrich, Steinheim, Germany
Antibody diluent (Dako REAL™)	DAKO Diagnostika, Hamburg, Germany
Avidin/biotin blocking Kit	Vector, Burlingame, USA
Beta mercapto ethanol	Sigma-Aldrich, Steinheim, Germany
Beriglobin®	Novartis, Marburg, Germany
Bovine serum albumine (BSA), pH 7.0	SERVA, Heidelberg, Germany
Brefeldin A	Sigma-Aldrich, Steinheim, Germany
Calcium Ionophore, sterile	Calbiochem-Merck, Darmstadt, Germany
Carboxy-fluorescein diacetate, succinimidyl ester (CFSE)	Molecular Probes Inc, Eugene, USA
Citric acid, $C_6H_8O_7$	Merck, Darmstadt, Germany
Complete Protease Inhibitor	Roche, Mannheim, Germany
CpG oligodeoxynucleotides	TIB MOLBIOL, Berlin, Germany
Cytofix™ Buffer	BD PharMingen, San Diego, USA
Detection System (Dako REAL, K 5005, Alkaline Phosphatase/RED)	DAKO Diagnostika, Hamburg, Germany
Developer, G153	AGFA, Cologne, Germany
n,n-Dimethyl formamide (DMF)	Sigma-Aldrich, Steinheim, Germany
Disodium hydrogen phosphat, Na_2HPO_4	Merck, Darmstadt, Germany
DNAse	Macherey-Nagel, Düren, Germany
Docosahexaenoic acid (DHA), $C_{22}H_{32}O_2$	Cayman Chemicals, Ann Arbor, USA
Docosahexaenoic acid (DHA), DHA500TG	CRODA, Nettetal, Germany
Docosahexaenoic acid (DHA), DHA700EE	CRODA, Nettetal, Germany
Diethanol amine, $(HOCH_2CH_2)_2NH$	Sigma-Aldrich, Steinheim, Germany
ECL High performance Chemiluminescence film	Amersham Buckinghamshire, United Kingdom
ECL Plus Western Blot Detection Reagents	Amersham, Buckinghamshire, United Kingdom
Extra Avidine Peroxidase	Sigma-Aldrich, Steinheim, Germany

FACS Flow	BD PharMingen, San Diego, USA
FastStart DNA Master SYBR® Green	Roche, Mannheim, Germany
Fetal calf serum (FCS)	Biochrom, Berlin, Germany
Ficoll, sterile, d = 1,077 g/mL	PAA, Pasching, Austria
Goat serum (DakoCytomation, X0907)	DAKO Diagnostika, Hamburg, Germany
L-Glutamine	Biochrom, Berlin, Germany
Glycine	SERVA, Heidelberg, Germany
Hydrochloric acid, HCl , 25%	Merck, Darmstadt, Germany
Hydrogen peroxide, H_2O_2, 30%	Merck, Darmstadt, Germany
Imject Alum	Amersham Pierce, Rockford, USA
Recombinant human Interleukin (IL)-4	Immunotools, Friesoythe, Germany
Kaiser's glycerol gelantine	Merck, Darmstadt, Germany
Ketamine hydrochlorid (Ketamine)	DeltaSelect, Dreiech, Germany
Lysing Solution	BD PharMingen, San Diego, USA
Magnesium chloride, $MgCl_2$	Merck, Darmstadt, Germany
Methanol C_2H_5OH	Merck, Darmstadt, Germany
Milk powder (Blotting Grade)	Roth, Karlsruhe, Germany
NE-PER™ Nuclear and Cytoplasmic Extraction Reagents	Pierce, Rockford, USA
Para-Nitrophenyl phosphate	Sigma-Aldrich, Steinheim, Germany
Nucleospin II Kit	Macherey-Nagel, Düren, Germany
Papanicolaou's solution (1a Harris' haematoxylin solution)	Merck, Darmstadt, Germany
Paraformaldehyde (PFA)	Sigma-Aldrich, Steinheim, Germany
Pellet Paint™ Co-Precipitant	Novagen, Madison, USA
Penicillin, 10.000 E	Biochrom, Berlin, Germany
Permeabilizing Solution (PermII)	BD PharMingen, San Diego, USA
Dulbecco´s Phosphate-Buffered Saline (PBS), without Ca^{2+}/Mg^{2+}, sterile	PAA, Pasching, Austria
Phorbol-12-myristate-13-acetate (PMA), sterile	Sigma-Aldrich, Steinheim, Germany
Phosphlow Perm Buffer	BD PharMingen, San Diego, USA
Physiological saline, 0.9 % NaCl	Braun, Melsungen, Germany
Potassium chloride, KCl	Merck, Darmstadt, Germany
Potassium dihydrogen phosphat, KH_2PO_4	Merck, Darmstadt, Germany

Precision Plus Protein Standard Dual	Bio-Rad, Munich, Germany
Propidium iodide	Sigma-Aldrich, Steinheim, Germany
Proteinase K	Macherey-Nagel, Düren, Germany
ProTaqs Tris, pH 7.6	BIOCYC, Luckenwalde, Germany
Rapid Fixer, G153	AGFA, Cologne, Germany
Reverse Transcription Kit	Applied Biosystems, Darmstadt, Germany
RPMI 1640, without Ca^{2+}/Mg^{2+}, sterile	Biochrom, Berlin, Germany
Saponin	Sigma-Aldrich, Steinheim, Germany
Sodium carbonate, Na_2CO_3	Merck, Darmstadt, Germany
Sodium chloride, NaCl	Merck, Darmstadt, Germany
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, Steinheim, Germany
Sodium hydrogen carbonate, $NaHCO_3$	Merck, Darmstadt, Germany
Staphylococcus Enterotoxine B (SEB)	Sigma-Aldrich, Steinheim, Germany
Streptavidin‐Horseradish Peroxidase (HRP), 100µg/mL	R&D, Minneapolis, USA
Streptavidin‐ Alkaline Phosphatase (AP)	ZYMED, San Francisco, USA
Streptomycin (10.000 g/ml)	Biochrom, Berlin, Germany
Sulphuric acid, H_2SO_4, 95‐97%	Riedel de Haen, Seelze, Germany
Tetramethyl benzidine dihydrochloride, TMB	Sigma-Aldrich, Steinheim, Germany
Tetramethyl ethylene diamine (TEMED), $C_6H_{16}N_2$	Bio-Rad, Munich, Germany
Toluidine blue O	Merck, Darmstadt, Germany
Tris(hydroxymethyl)aminomethane (Tris-Base)	Sigma-Aldrich, Steinheim, Germany
Tween20	Bio-Rad, Munich, Germany
Xylazine hydrochlorid (Rompun)	Bayer, Leverkusen, Germany
Western Blot Loading Buffer	DRFZ
WST-1 reagent	Boehringer Mannheim, Germany

Commodities and labware

Commodities		Manufacturer
6 Well plates, sterile	Suspension	Greiner, Essen, Germany
24 Well plates, sterile	Tissue, suspension	Greiner, Essen, Germany
48 Well plates, sterile	Tissue, suspension	Greiner, Essen, Germany
96 Well plates, sterile	Tissue, suspension	Greiner, Essen, Germany
96 Well plates (ELISA)	Maxi sorb	NUNC, Wiesbaden, Germany
96 Well plates, sterile (ELIspot)	0.45 µm Hydrophob High Protein Binding Immibilion-P Membrane	Millipore, Schwallbach, Germany
Biopsy punch, sterile	4. 5 and 6 mm diameter	Stiefel , Offenbach, Germany
Cohesive conforming bandage	Peha-haft, 6 cm width	Hartmann, Heidenheim, Germany
Cover slips		Menzel-Gläser, Braunschweig, Germany
Cryo spray	Instant freezing spray	Bio-optica, Milan, Italy
Disposable hospital razor		Wilkinson, Solingen, Germany
Disposable vinyl specimen	Cryomold Tissue-Tek	Sakura, Torrance, USA
Finn chambers		Epitest Ltd Oy, Tuusula, Finland
Filter paper discs for Finn chambers	7.5 mm diameter	Epitest Ltd Oy, Tuusula, Finland
Hamilton Pipette		Hamilton Company, Reno, USA
Hypodermic needle, sterile	0,45 x 25 mm, 26G x 1', Sterican 100	Braun, Melsungen, Germany
Light Cycler Capillaries		Roche, Mannheim, Germany
Liquid Blocker	Pap Pen	Kisker-Biotech, Steinfurt, Germany
Microscope slides	Super frost plus R.	Langenbrinck, Emmendingen, Germany
O.C.T. medium	Tissue-Tek	Sakura, Torrance, USA
Ophthamic gel	Vidisic	Bausch & Lomb, Berlin, Germany
Protean3 system		Bio-Rad, Munich, Germany
Scalpel, sterile	No. 20	Feather, Osaka, Japan

Syringes, sterile	10ml, 20 ml, 50ml	Braun, Melsungen, Germany
Wattman Paper		Schleicher & Schull, Dassel, Germany
Western Blot Cassette	ECL Hypercassette	Amersham Buckinghamshire, United Kingdom
Western Blot Membrane	Polyvinylidenfluorid (PVDF)	Amersham Buckinghamshire, United Kingdom

Reaction tubes, pipette tips and other commodities were used from Eppendorf, Falcon, and Sarstedt.

Equipment

Equipment	Model	Manufacturer
Autoclave		MELAG, Berlin, Germany
Camera	EOS 20D	Canon, Krefeldt, Germany
Centrifuge	Megafuge 1.OR	Heraeus, Hanau, Germany
	Multifuge 4KR	Heraeus, Hanau, Germany
	5417R; 5417R	Eppendorf, Hamburg, Germany
	Minifuge RF	Heraeus, Hanau, Germany
Cryostat	Jung frigocut 2800N	Leica, Wetzlar, Germany
Electronic cell counter	CASY 1, Modell TT	Innovatis, Reutlingen, Germany
ELISA reader	Dynex MRX version 1.33	DYNATECH, Chantilly, USA
ELISPOT reader	CTL ImmunoSpot® S4 Analyzer	C.T.L Cellular Technology, Cleveland, USA
Flow bench	HERA safe	Heraeus, Hanau, Germany
Flow cytometre	FACS Calibur	Becton Dickinson, Heidelberg, Germany
Freezer (-20°C)/Fridge (4°C)	TKF380	EUREKA, Emsdetten, Germany
Freezer (-80°C)	Hera Freeze	Heraeus, Hanau, Germany
Gel chamber 40	1214 peqLab	PEQLAB Biotechnologie, Erlangen, Germany
Gradient cycler	Px2 Thermal Cycler	Thermo, Hamburg, Germany
Hot plate	nuova II	Thermolyne, Dubuque, USA
Incubator	HERA cell	Heraeus, Hanau, Germany
Lab balance		Sartorius, Göttingen, Germany
Light Cycler	LightCycler 1.5	Roche, Mannheim, Germany
Magnetic cell sorter	autoMACS Separator	Miltenyi Biotec, Bergisch Gladbach, Germany
Magnetic stirrer	Magnetmix 2070	Hecht-Assistent, Sondenheim, Germany
Microscope	Axioskop and Axioplan2 imaging	Carl Zeiss, Jena, Germany
Microscope camera	Axio Cam HRc	Carl Zeiss, Jena, Germany
Pipets	10µl, 100µl, 200µl;	Eppendorf, Hamburg, Germany

	1000µl; multichannel, multistepper	
Pipettors	Pipetus	Hirschmann Laborgeräte, Eberstadt, Germany
pH electrode		NEOLAB, Heidelberg, Germany
pH-metre	MV 870 Digital	PRÄCITRONIC, Dresden, Germany
Power Supply	PowerPac300	Bio-Rad, Munich, Germany
Rotator	Coulter Mixer	Denleytech, Woking, United Kingdom
Shaker	IKA-VIBRAX-VXR	IKA, Staufen, Germany
Spectrophotometre	ND-1000	NanoDrop, Wilmington, USA
Ultrasonic bath	Sonorex TK52	Bandelin, Berlin, Germany
Vortex	REAX 2000	Heidolph, Schwabach, Germany
Water bath	GFL1092	JULABO, Seelbach, Germany

Software

Software	Manufacturer
Axio Vision LE Application 4.5.0.0	Carl Zeiss, Jena, Germany
ImmunoSpot v4.0.13	C.T.L Cellular Technology, Cleveland, USA
Light Cycler Software Version 3	Roche, Mannheim, Germany
NCBI databases	National Center for Biotechnology Information Bethesda, USA
ND-1000	NanoDrop, Wilmington, USA
Primer 3	SourceForge, Mountain View, USA
Revelation G3.2	DYNEX, Berlin, Germany
SPSS (Statistical Package for the Social Sciences) for Windows, Release 12.0 & 14.0	SPSS Inc., Chicago, USA
UCSC databases	UCSC Genome Bioinformatics Group, Santa Cruz, USA

Buffers and solutions

ACE-DMF buffer (pH 5.0)	64 mM Sodium acetate 45 mM Acetate
Blotting buffer	25 mM Tris-base 190 mM glycine 20 % v/v Methanol
Diethanol amine buffer (pH 9.9)	840 mM Diethanol amine 1.1 mM $MgCl_2$
FACS buffer	PBS 1 % BSA
MACS buffer	PBS 20 mM EDTA 0.2 % BSA
Medium complete	500 mL RPMI1640, with 2.0 g/L $NaHCO_3$, with stabile glutamine 50 mL FCS 100 U/mL Penecillin 100 µg/mL Streptomycin
Ketamine / Rompun (8 : 1 : 1)	0.9 % NaCl : 50 mg/mL Ketamine 2 % Rompun
Running buffer	25 mM Tris-base 190 mM Glycine 7 mM SDS

Separating gel (5 %)	5 % v/v Acryl amide 0.125 M Tris Buffer 0.1 % v/v SDS 0.1 % v/v APS 0.01 % v/v TEMED
Sodium carbonate buffer (pH=9.0)	45 mM $NaHCO_3$ 9.1 M Na_2CO_3
Stacking gel	12 % v/v Acryl amide 0.5 M Tris Buffer 0.1 % v/v SDS 0.1 % v/v APS 0.04 % v/v TEMED
TMB buffer (pH=5.5)	51.4 mM Na_2HPO_4 24.3 mM citric acid
Tris-buffered saline (TBS)	20 mM Tris-Base 75 mM NaCl

List of abbreviations

AA	Arachidonic acid, 20:4n-6
AICD	Activation induced cell death
AID	Activation induced (cytidine) desaminase
ALA	α-linolenic acid, 18:3n-3
AP-1	Activator protein 1
APC	Antigen presenting cell
BMI	Body mass index
C_H	Heavy chain constant regions
CLA	Cutaneous lymphocyte-associated antigen
COX	Cyclooxygenase
CSS	Clinical skin score
CSR	Class switch recombination
DAG	Diacylglycerol
DC	Dendritic cells
DGLA	Dihomo-gamma linolenic acid, 20:3n-6
DHA	Docosahexaenoic acid, 22:6n-3
e.c.	Epicutaneous
εGLT	Epsilon germline transcript
ELISA	Enzyme Linked Immuno Assay
ELIspot	Enzyme Linked Immuno Spot Technique
EPA	Eicosapentaenoic acid, 20:5n-3
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorter
FcεRI	High affinity receptor for IgE
FLG	Filament-aggregating protein
FSC	Forward light scatter
g	Acceleration of gravity
GCK	Germinal center kinase
GLA	γ-linoleic acid, 20:3n-6
HDL	High density lipoprotein
HLA	human leukocyte antigen
HPRT	Hypoxanthine-Guanine Phosphoribosyltransferase
Ig	Immunoglobulin

IDEC	Inflammatory dendritic epidermal cells
IFN	Interferon
IκB	inhibitor of NFκB
IKK	inhibitor of NFκB (IκB) kinase
IL-	Interleukin
IL-4R	Interleukin-4 Receptor
i.p.	Intraperitoneal
IRS-	Insulin receptor substrate
ITIM	Immuno-tyrosine inhibitory motif
JAK	Janus activated kinase
JNK	c-jun kinase
LA	Linoleic acid, 18:2n-6
LC	Langerhans cells
LOX	Lipoxygenase
LT	Leukotriene
LXR	Liver X receptor
MACS	Magnetic cell sorting
MAPK	Mitogen-activated protein kinase
MEKK1	MAPK/ ERK kinase kinase 1
MFI	Mean fluorescence intensity
MHC II	Major histocompatibility complex II
min	Minute
MO	Monocyte
n.d.	Not detectable
NFκB	Nuclear factor kappa B
NIK	NFκB inducing kinase
n.s.	Not significant
OA	Oleic acid, 18:1n-9
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cells
PG	Prostaglandin
PI_3-K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PL	Phospholipase
PPAR	Peroxisome proliferator-activated receptor

pSTAT6	Phosphorylated signal transducer and activator of transcription 6
PUFA	Polyunsaturated fatty acids
RT-PCR	Reverse-transcriptase polymerase chain reaction
RXR	Retinoic acid X receptor
S. aureus	Staphylococcus aureus
SCORAD	Severity Scoring of Atopic Dermatitis
SH2	Src homology domain 2
Shp-1	SH2-containing tyrosine phosphatase--1
SREBP	Sterol regulatory-element binding protein
SSC	Side light scatter
STAT6	Signal transducer and activator of transcription 6
$T_H1/2$	T_{Helper} cell type 1/2
TNF	Tumor necrosis factor
TRAF	TNF-receptor-associated factor
T_{reg}	Regulatory T cells
TSLP	Thymic stromal lymphopoietin
TX	Thromboxane

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Statement of authorship

I hereby certify that I performed and wrote the current thesis independently and did not use other than the listed support. This thesis does not exist neither in the same or similar form nor is it submitted to another examination procedure. I did not gain an academic degree before nor did I try to do this. I claim full responsibility for the contents of this thesis.

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